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(54) **Title:** NITROSYLATED BLOOD SUBSTITUTES

(57) **Abstract**

Blood substitutes to which are directly or indirectly linked an NO or NO_2 group, particularly heme-containing proteins. Pharmaceutical compositions comprising the above and pharmaceutical carriers, and their uses as therapeutic agents.

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NITROSYLATED BLOOD SUBSTITUTES

This application is a continuation-in-part of U.S. Application Serial No. 08/198,854, filed February 17, 1994; which is a divisional of U.S. Application Serial No. 07/943,835, filed September 14, 1992 (now abandoned); which is a continuation-in-part of U.S. Application Serial No. 07/791,668, filed November 14, 1991 (now abandoned).

The need for an artificial blood substitute has become increasingly compelling because of periodic shortages of blood, and the rising incidence of blood-borne illnesses such as AIDS and hepatitis. Notwithstanding the efforts of numerous investigators there are substantial obstacles that need to be overcome before any such product gains widespread use. Recent clinical trials have been terminated prematurely due to gastrointestinal, cardiovascular, and respiratory side effects; specifically, increases in blood pressure, painful abdominal symptoms, and arterial oxygen desaturation. Hemoglobin and other blood substitutes and oxygen delivery systems cause vascular constriction in the entire cardiovascular system, particularly in the lung and gastrointestinal tract by causing the contraction of vascular smooth muscle. Further, hemoglobins and other oxygen delivery systems that consume or scavenge nitric oxide

thereby cause vasoconstriction which hinders oxygen delivery and thereby raises blood pressure. Overcoming these limitations would end a lengthy quest for an alternative to red blood cell transfusions and satisfy a strong medical need.

Several complications of blood substitute therapy, such as renal toxicity, short half-life in the circulation and excessively high oxygen affinity have been ameliorated by rational approaches to drug design, such as cross-linking of lower molecular weight dimers and subunits. The residual side effects of cell-free hemoglobin solutions appear to be the direct consequence of their high affinity binding of nitric oxide (NO). In this respect, NO produced locally in blood vessels, airways and the gastrointestinal tract regulates the pressure of the blood, airway tone, and the peristaltic contractions of the stomach, intestines and colon. Both enzyme inhibitors that selectively decrease NO production and hemoglobin which traps nitric oxide, promote vasoconstriction, alterations in intestinal motility, and ventilation perfusion mismatching in animal models.

Cell-free blood substitute compositions sought to be used for treating hemorrhage and preserving organs and tissue for transplantation have predominantly been preparations of heme-containing proteins. Numerous investigators and foreign and domestic companies have sought to produce isolated human, bovine and other hemoglobin and modified hemoglobins for use in cell-free blood substitute preparations. Native hemoglobin has been isolated from blood and used in such preparations.

Modified hemoglobins have been prepared and used for such cell-free preparations. For example, cross-linked polymerized hemoglobins (see U.S. Patent No. 5,194,590) and

cross-linked polymerized pyridoxylated hemoglobin (U.S. Patent No. 5,194,590 and 4,826,811) have been prepared. Multimeric hemoglobin-like proteins based on pseudo-tetramer containing pseudo-dimer polypeptides with globin-like domains have been used in order to prolong hemoglobin half life (see WO 93/09143). Variants such as conjugates of hemoglobin with other drugs are also used (see WO 93/088422). Di-alpha-globin-like polypeptide and di-beta-globin-like polypeptides connected into a single chain and incorporating heme have been used to prepare a human hemoglobin-like protein (see WO 90/13645). Hemoglobin mutants having lower O-affinity have been prepared by recombinant techniques and used as blood substitutes (see WO 88/09179).

Numerous other modifications of hemoglobins are disclosed in the patent literature for use as oxygen carrying compounds in blood substitutes. Hemoglobin and heme-containing protein based blood substitute compositions are disclosed, *inter alia*, in U.S. Patent Nos. 5,217,648; 5,194,590; 5,061,688; 4,826,811;5,281,579; 5,128,452; 5,248,766; 5,041,615; 4,861,867; 4,831,012; 5,296,465; 5,084,558; 5,295,944; 4,780,210; 4,925,574;5,264,555 and numerous others; and in PCT publication nos. WO 93/09143; WO 93/08842; WO 90/13645; WO 92/02239; WO 89/12456; WO 88/03408; WO 92/20369; WO 92/09630; WO 91/07190 and numerous others.

Another principal type of cell-free blood substitute compositions are oxygen carrying emulsion compositions of fluorocarbon particles. Typical particle sizes are 0.05 to 0.3 mu. Perflurohydrocarbons are well known oxygen carriers for artificial blood and *in vitro* perfusion fluids. The perflurocarbon compounds used can generally be of about 3-15 carbon atoms, particularly mixtures of them. Examples include perfluoro-decalin, -methyldecalin, -(3-5C) alkylcyclohexanes, -(5-7C)

alkytertrahydrofurans, -(4-6C) alkyltetrahydropyrans and (9-11C) alkanes, perfluoromethyladamantane, perfluorodimethyladamantane, perfluoroethylmethyladamantane, perfluorodiethyladamanatane and perfluoroadamantane.

These compositions can also use perfluorotertiaryamines. Examples of these are perfluoro - (9-11C) tert, alkylamines, -N-(4-6C) alklpiperidines or -N-(5-7C)alkylmorpholines, particularly perfluoro-N,N-dibutylmethylamine.

These compositions can further include nonionic surfactants, (e.g., a polyoxyethylene - polyoxypropylene copolymer) phospholipids, polymeric emulsifiers and fatty acids.

This invention relates to nitrosylation of blood substitutes, particularly including heme proteins such as hemoglobin as a therapeutic modality. The invention also relates to nitroso-protein compounds and their use as a means to selectively regulate specific protein functions, to selectively regulate cellular function, to endow the protein with new smooth muscle relaxant and platelet inhibitory properties and to provide targeted delivery of nitric oxide to specific bodily sites.

An additional aspect of the invention is blood substitute compositions comprising nitrosylated proteins and other constituents. These blood substitute compositions are preferably cell free. Principally useful in such compositions are various forms of nitrosylated hemoglobin and modified hemoglobins.

These nitrosylated blood substitutes have a vasorelaxant activity which is directly opposite to the

vasoconstrictive properties of the non-nitrosylation forms. The administration of the nitrosylated forms effect vasodilation both by adding the administered nitric oxide to the recipient but also by not reducing through scavenging, the constitutive levels.

Additionally, the invention relates to nitrosylation of sites such as sulfhydryl (thiol), oxygen, carbon and nitrogen, present on proteins and amino acids, as a means to achieve the above physiological effects. The therapeutic effects may be achieved by the administration of nitrosylated proteins and amino acids as pharmaceutical compositions, or by nitrosylation of proteins and amino acids *in vivo* through the administration of a nitrosylating agent, such as in the form of a pharmaceutical composition.

Additionally, the invention provides a method for inhibiting the vasoconstrictive and nitric oxide depleting effects of hemoglobin and heme-containing based blood substitute compositions by the concurrent systemic administration of nitric oxide or a compound which donates, releases or transfers nitric oxide. Such administration can, for example, be by inhalation or intravenously, separately or in composition with the blood substitute.

NO has been shown to exert a wide variety of biological effects. In the context of this application, vasodilation, bronchodilation and inhibition of intestinal and sphincteric motility are noteworthy. Moreover, NO has also been implicated in the pathogenesis of inflammation, cellular dysfunction and toxicity when produced in settings of oxidant stress. These divergent effects in different biological systems are achieved via a rich redox and additive chemistry of the molecule. Both covalent modifications of proteins as well as oxidation events that do not involve

attachment of the NO group, have been adopted as signalling mechanisms.

In physiological systems, NO reacts with O_2^-, O_2, and transition metals. Each of the products of these reactions - peroxynitrite ($OONO^-$), NO_x and metal-NO adducts, respectively - support additional nitrosative and oxidative reactions with thiol groups. Accordingly, thiol and metal-containing proteins are the major target sites for NO. These targets include plasma proteins (infra), membrane-associated signalling proteins, ion channels and receptors. Furthermore, several enzymes containing either thiols or metals are known to be NO-responsive. In particular, NO activates the prototypic heme containing enzyme guanylyl cyclase, which elicits many target cell responses, including smooth muscle relaxation. Heme proteins also serve in the biometabolism of NO. For example, inactivation of NO in the blood, and the tissues it perfuses is achieved by interactions with hemoglobin. Unlike CO and O_2, nitric oxide binds effectively to both ferric (III) and ferrous (II) heme. The ratio of rates of NO uptake and release for Fe(II)-hemoglobin is five-six orders of magnitude greater than that of O_2, largely due to the marked differences in ligand dissociation rates (~ 10^{-5} s^{-1} for NO and ~ 20 s^{-1} for O_2). The Fe(III) NO-heme adduct exhibits more rapid loss of NO. No is also inactivated rapidly by O_2hemoglobin, forming methemoglobin and nitrate.

The reaction between low molecular weight thiols, such as cysteine, homocysteine, and N-acetylcysteine, and nitric oxide (NO) has been studied in biological systems. NO has been shown to induce relaxation of vascular smooth muscle, and inhibition of platelet aggregation, through activation of guanylate cyclase and elevation of cyclic GMP levels. Evidence exists that low molecular weight thiols

react readily with NO to form S-nitrosothiols, which are significantly more stable than NO itself, and act as potent vasodilators and platelet inhibitors. These adducts have also been proposed as biologically active intermediates in the metabolism of organic nitrates (Ignarro *el al., J. Pharmacol. Exp. Ther.* 218:739 (1981); Mellion, *et al., Mol-Pha ol. 23:653* (1983); Loscalzo, *et al, J. Clin. Invest. 76:966* (1985)).

We have previously espoused that the biological chemistry of NO involves an array of redox related forms: NO^+, $NO^\bullet$, and NO^-. The distinctive properties and differential reactivities of these species is critical to the elucidation of the biological actions of "nitric oxide". Whereas $NO^\bullet$ shows preferential reactivity towards metal centers, species with NO^+ character target sulfhydryl groups, forming S-nitrosothiols (RS-NO). Intriguingly, hemoglobin contains a highly reactive β93 SH group (pK of 5.5), making it susceptible to S-nitrosylation. Since the NO group possesses NO^+ character it is well shielded from the heme center of the molecule (which binds $NO^\bullet$).

Simulation of the diffusion and reaction of endogenously produced nitric oxide has lead to the paradoxical conclusion that any $NO^\bullet$ produced should be rapidly scavenged in the vasculature by hemoglobin, leading to increases in blood pressure (and related clinical manifestations).

Many proteins of physiological significance possess intramolecular thiols in the form of cysteine residues. These thiol groups are often of critical importance in the functional properties of such proteins. These sulfhydryl groups are highly specialized and utilized extensively in physiological processes such as metabolic regulation,

structural stabilization, transfer of reducing equivalents, detoxification pathways and enzyme catalysis (Gilbert, H.F., "Molecular and Cellular Aspects of Thiol-Disulfide Exchange", *Advances in Enzymology*, A. Miester, J. Wiley & Sons, Eds. New York 1990, pages 69-172.)

Thiols are also present on those proteins the function of which is to transport and deliver specific molecules to particular bodily tissues. For example, lipoproteins are globular particles of high molecular weight that transport nonpolar lipids through the plasma. These proteins contain thiols in the region of the protein which controls cellular uptake of the lipoprotein (Mahley *et al. JAMA* 265:78-83 (1991)). Hyper-lipoproteinemias, resulting from excessive lipoprotein (and thus, lipid) uptake, cause life-threatening diseases such as atherosclerosis and pancreatitis.

The thiol contained in hemoglobin regulates the affinity of hemoglobin for oxygen, and thus has a critical role in the delivery of oxygen to bodily tissues. The reaction between the free NO radical occurs at the iron-binding site of hemoglobin, and not the thiol. As a result, methemoglobin is generated, which impairs oxygen-hemoglobin binding, and thus, oxygen transport. Other proteins such as thrombolytic agents, immunoglobulins, and albumin, possess free thiol groups that are important in regulating protein function.

Protein thiols may, under certain pathophysiological conditions, cause a protein to exert a detrimental effect. For example, cathepsin, a sulfhydryl enzyme involved in the breakdown of cellular constituents, is critically dependent upon sulfhydryl groups for proteolytic activity. However, uncontrolled proteolysis caused by this

enzyme leads to tissue damage; specifically lung damage caused by smoking.

The reaction between NO and the thiols of intact protein molecules has previously been studied only to a very limited extent. There is some evidence for the reaction between proteins and nitro(so)-containing compounds *in vivo*. Investigators have observed that the denitrification of nitroglycerin in plasma is catalyzed by the thiol of albumin (Chong *et al., Drug Met. and Disp. 18:61* (1990), and these authors suggest an analogy between this mechanism and the thiol-dependent enzymatic denitrification of nitroglycerin with glutathione S-transferase in a reaction which generates thionitrates (Keene *et al., JBC* 251:6183 (1976)). In addition, hemoproteins have been shown to catalyze denitrification of nitroglycerin, and to react by way of thiol groups with certain nitroso-compounds as part of the hypothesized detoxification pathway for arylhydroxylamines (Bennett *et al., J. Pharmacol. Exp. Ther. 237:629*(1986); Umemoto *et al., Biochem. Biophys. Res. Commun.* 151:1326 (1988)). The chemical identity of intermediates in these reactions is not known.

Nitrosylation of amino acids can also be accomplished at sites other than the thiol group. Tyrosine, an aromatic amino acid, which is prevalent in proteins, peptides, and other chemical compounds, contains a phenolic ring, hydroxyl group, and amino group. It is generally known that nitration of phenol yields ortho-nitrophenyl and para-nitrophenyl C-nitrosylation products. Nitrosylation of tyrosine, using nitrous acid, has been shown to yield C-nitrosylated tyrosine (Reeve, R.M., *Histochem. Cytochem.* 16(3):191-8 (1968)), and it has been suggested that this process produces O-nitrosotyrosine as a preliminary product which then rearranges into the C-nitrosylated product.

(Baliga, B.T. *Org. Chem.* 35(6):2031-2032 (1970); Bonnett *et al.*, J.C.S. Perkin Trans. 1:2261-2264 (1975)).

In general, synthesis of polynitrosated peptides and proteins can be achieved in several ways. 1) Mono S-nitrosylation is best achieved by incubating peptides and proteins (in deionized water in an equimolar concentration of acidified nitrite (final concentration 0.5 N HCL) for a period of 1-30 minutes. The incubation time depends on the efficiency of nitrosation and the tolerance of the protein. Nitrosation can also be achieved with a variety of other nitrosating agents including compounds such as S-nitrosocysteines, S-nitrosoglutathione and related alkyl nitrites. These compounds are used when the peptide or protein does not tolerate harsh acidic conditions.

There are two ways of achieving poly S-nitrosation. In the first, the peptide or protein is reduced in 100-1000 molar excess dithiothreitol for 30-60 minutes. This exposes intramolecular thiols. The peptide or protein is separated from dithiothreitol by gel filtration (G-25). The protein is then exposed to increasing concentrations of acidified nitrite (0.5 N HCl) in relative excess over protein. Complementary measurements of Saville indicate when S-nitrosation is complete. For example, with albumin, this procedure leads to approximately 20 intramolecular S-NO derivatives.

Alternatively, the protein can be treated with thiolating agent such as homocysteine thiolactone. This tends to add homocystine groups to exposed amine residues in proteins. The derivatized protein can then be S-nitrosated by exposure to acidified nitrite. Exposure to incerasing concentrations of nitrite with complementary measurements of Saville can be used to ascertain when S-nitrosation is

maximal. Alternatively, thiol groups can be quantified on the protein using standard methodologies and then the protein treated with a stoichiometric concentration of acidified nitrite (0.5 N HCl).

Polynitrosation of nucleophilic functional groups (other than thiol) can be achieved when proteins are incubated with excess acidified nitrite. The order of protein reactivity is tyrosine followed by amines on residues such as trytophan. Amide linkages are probably less reactive. Accordingly, many NO groups can be added to proteins by simply incubating the protein with high excess acidified nitrite. For example, exposure of albumin to 1000 fold excess nitrite leads to approximately 200 moles of NO/mole protein. These experiements are performed in 0.5 normal HCl with incubations for approximately one hour. ^{15}N NMR can be used to determine where the addition (or substitution) by NO takes place.

Further, nitrosation can be achieved by exposure to authentic nitric oxide gas under anaerobic conditions. For successful nitrosation proteins should be incubated in at least 5 atmospheres of NO gas for several hours. Incubation time is protein specific. This can lead to NO attachment to a variety of protein bases. Best characterized reactions involve primary amines. This mechanism provides a pathway to sustain N-nitrosation reactions without deamination. Specifically, exposure to acidified nitrite would otherwise lead to deamination of primary amines whereas this method leads to formation of N-hydroxynitrosamines with potent bioactivity. Similar substitutions at other basic centers also occur.

The chemistry of amino acid side chains, such as those found on tyrosine and other aromatic amino acids, has

a critical role in ensuring proper enzymatic function within the body. In addition, the hydroxyl group of tyrosine plays a central role in a variety of cell regulatory functions, with phosphorylation of tyrosine being one such critical cell regulatory event. In addition to possessing bioactive side chains, these aromatic amino acids serve as precursors to numerous important biomolecules such as hormones, vitamins, coenzymes, and neurotransmitters.

The current state of the art lacks chemical methods for modifying the activity and regulating the intermediary cellular metabolism of the amino acids and proteins which play a critical role in biological systems. Moreover, the ability to regulate protein function by nitrosylation was, prior to the present invention, unappreciated in the art.

It is appreciated in the art that, as a result of their increased molecular weight and tertiary structure, protein molecules differ significantly from low molecular weight thiols. Furthermore, because of these differences, it would not be expected that protein thiols could be successfully nitrosylated in the same manner as low molecular weight thiols, or that, if nitrosylated, they would react in the same manner. Furthermore, it would be equally unexpected that nitrosylation of additional sites such as oxygen, carbon and nitrogen would provide a means for regulation of protein function.

Because of the great importance of diverse proteins and amino acids in all biological systems, it would be extremely desirable to have a method for achieving selective regulation of protein and amino acid function. There are virtually unlimited situations in which the ability to regulate amino acid or protein function by nitrosylation would be of tremendous therapeutic significance. Examples of

ways in which regulation or modification of function could be achieved would be the following: (1) To enhance or prolong the beneficial properties of the protein or amino acid; (2) to imbue the protein or amino acid with additional beneficial properties; (3) to eliminate detrimental properties of a protein or amino acid; and (4) to alter the metabolism or uptake of proteins or amino acids in physiological systems.

The present invention represents a novel method for achieving these therapeutically significant objectives by regulation of protein and amino acid function with either of the following methods: (1) administration of particular nitrosylated proteins or amino acids to a patient; and (2) nitrosylation of a protein or amino acid *in vivo* by the administration of a nitrosylating agent to a patient. In addition, the invention represents the discovery of exemplary S-nitroso-proteins and amino acids of great biological and pharmacological utility.

This invention is based on the discovery by the inventors that nitrosylating thiols, as well as oxygen, carbon and nitrogen present on proteins and amino acids provides a means for achieving selective regulation of protein and amino acid function. This concept can be employed to generate S-nitroso-protein compounds, as well as other nitrosylated proteins and amino acids, which possess specific properties, and can be directly administered to a patient. In the alternative, the invention provides a means for *in vivo* regulation of protein or amino acid function by nitrosylation. The invention is therefore directed to novel S-nitroso-proteins and the therapeutic uses thereof, as well as the nitrosylation of proteins *in vivo*, as a therapeutic modality. The invention is also directed to nitrosylation of oxygen, carbon and nitrogen sites of proteins and amino acids, as a therapeutic modality.

In one aspect this invention is directed to compounds comprising enzymes, nitrosylated thrombolytic agents, particularly nitrosylated thrombolytic enzymes. Such enzymes include tissue-type plasminogen activator, streptokinase, urokinase and cathepsin and others known or yet to be discovered.

This invention is also directed to compounds comprising S-nitroso-lipoprotein. Lipoproteins which may be contained in the compound include chylomicrons, chylomicron remnant particles, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) high-density lipoprotein (HDL) and lipoprotein (a).

This invention is also directed to compounds comprising S-nitroso-immunoglobulin. Immunoglobulins contained in this compound include IgG, IgM, IgA, IgD, IgE.

This invention is also directed to compounds comprising a blood substitute which is directly or indirectly linked to an NO or NO_2 group. The blood substitute is, in one preferred embodiment, a heme protein, particularly a hemoglobin such as human or bovine hemoglobin. The blood substitute compound of the invention also includes modified hemoglobins described in the background literature which have been nitrosylated by direct or indirect linking thereto of an NO or NO_2 group. This NO or NO_2 group can be, for example, attached at a site consisting of an S-nitroso, N-nitroso, O-nitroso and C-nitroso moiety. The invention is also directed to the compound S-nitroso-hemoglobin.

The invention is also directed to the compound S-nitroso-myoglobin.

The invention is also directed to pharmaceutical compositions containing the compounds of the invention, together with a pharmaceutically acceptable carrier.

The invention is particularly directed to blood substitute compositions comprising the nitrosylated derivative compounds of blood substitute compounds identified above. As such the composition preferably includes nitrosylated heme proteins particularly nitrosylated hemoglobin and modified hemoglobins whether of human or animal origin. Also contemplated are heme containing proteins such as hemoglobin which are made by recombinant methods or are synthesized chemically.

The invention also contemplates a composition containing the nitrosylated blood substitute compounds of the invention and which further include an additional component selected from the group consisting of a nonionic surfactant, a phospholipid, an emulsifier and a fatty acid.

The invention also provides a blood substitute composition comprising an emulsion of perfluorocarbon particles in a pharmaceutically acceptable carrier and which further comprises nitric oxide or a compound capable of donating, releasing or transferring nitric oxide. This compound is preferably a nitrosothiol.

The invention is also directed to a method for regulating oxygen delivery to bodily sites by administering pharmaceutical compositions containing S-nitroso-hemoglobin and S-nitroso-myoglobin.

The invention also relates to methods for effecting vasodilation, platelet inhibition, and thrombolysis; and for treating cardiovascular disorders, comprising administering

the pharmaceutical compositions of the invention to an animal.

This invention is also directed to a method for effecting platelet inhibition, comprising administering a pharmaceutical composition comprised of S-nitroso-albumin. An additional embodiment of the invention comprises the method for causing relaxation of airway smooth muscle and for the treatment or prevention of respiratory disorders, comprising administering a pharmaceutical composition containing S-nitroso-albumin.

This invention also is directed to a method for causing vasodilation, platelet inhibition and thrombolysis, comprising administering a nitrosylating agent to an animal.

This invention also is directed to a method for regulation of protein function *in vivo*, comprising administering a nitrosylating agent to an animal.

The invention is also directed to a method for preventing the uptake of a protein by cells, comprising administering a nitrosylating agent to a patient.

The invention is also directed to a method for causing relaxation of non-vascular smooth muscle, comprising administering the pharmaceutical compositions of the invention to an animal.

The invention is also directed to a method for regulating the function of proteins in which the thiol is bound to a methyl group, comprising the steps of removing the methyl groups from the protein by selective de-methylation, and reacting the free thiol group with a nitrosylating agent.

The invention is also directed to a method for regulating the function of a protein which lacks a free thiol group, comprising the steps of adding a thiol group to the protein, and reacting the thiol group with a nitrosylating agent.

The invention is also directed to a method for regulating cellular function, comprising the S-nitrosylation of a protein which is a cellular component or which affects a cellular function.

The invention is also directed to a method for delivering nitric oxide to specific, targeted sites in the body comprising administering an effective amount of the pharmaceutical compositions of the invention to an animal.

The invention is also directed to a method for inhibiting platelet function, comprising the nitrosylation of a protein or amino acid at other sites, in addition to thiol groups, which are present on said protein or amino acid.

The invention is also directed to a method for causing vasodilation, comprising the nitrosylation of a protein or amino acid at other sites, in addition to thiol groups, which are present on said protein or amino acid.

The invention is also directed to a method for relaxing smooth muscle, comprising the nitrosylation of a protein or amino acid at other sites, in addition to thiol groups, which are present on said protein or amino acid.

The invention is also directed to a method for regulating cellular function, comprising the nitrosylation of a protein or amino acid at other sites, in addition to thiol groups, which are present on said protein or amino acid.

The invention is also directed to a method for delivery of nitric oxide to specific, targeted sites in the body, comprising the nitrosylation of a protein or amino acid at other sites, in addition to thiol groups, which are present on said protein or amino acid.

The other sites which are nitrosylated are selected from the group consisting of oxygen, carbon and nitrogen.

The invention is also directed to a method for inhibiting platelet function, comprising administering a pharmaceutical composition comprised of a compound selected from the group consisting of any S-nitroso-protein.

The invention is also directed to a method for causing vasodilation, comprising administering a pharmaceutical composition comprised of a compound selected from the group consisting of any S-nitroso-protein.

The invention is also directed to a method for treatment or prevention of cardiovascular disorders, comprising administering a pharmaceutical composition comprised of a compound selected from the group consisting of any S-nitroso-protein.

The invention is directed to a method for relaxing non-vascular smooth muscle, comprising administering a pharmaceutical composition comprised of a compound selected from the group consisting of any S-nitroso-protein.

The invention is also directed to a method for treatment or prevention of respiratory disorders, comprising administering a pharmaceutical composition comprised of a compound selected from the group consisting of any S-nitroso-protein.

The invention is also directed to a method for delivering nitric oxide to specific, targeted sites in the body, comprising administering a pharmaceutical composition comprised of a compound selected from the group consisting of any S-nitroso-protein.

Figure 1. S-NO-t-PA spectroscopy (Example 1.B.2).

1a: The ultraviolet absorption spectrum S-NO-t-PA (15μM) relative to unmodified t-PA.

1b: The chemical shift of S-[15 N]O-t-PA (35 μM) at 751 ppm relative to nitrite using [^{15}N]NMR.

Figure 2. Determination of S-NO bond formation in the synthesis of S-NO-t-PA (Example 1.B.2).

Figure 3. [^{15}N]-NMR Spectrum of [^{15}N]-labeled S-nitroso-BSA (Example 2.B).

Figure 4. Concentration-dependent binding of t-PA and S-NO-tPA to fibrinogen-coated wells (Example 6.A.1).

Figure 5. Double reciprocal plots for S-NO-t-PA. Results are expressed as mean ± S.D. (n = 3).

5a: Double reciprocal plot 1/v versus 1/s for t-PA and SNO-t-PA generated against the chromogemc substrate S2288 (Example 6.A.2).

5b: The curves for activation of glu-plasminogen (0.1-10 μM) by t-PA and S-NO-t-PA, generated using the plasmin-specific chromogenic substrate S2251 (Example 6.A.2).

Figure 6. Fibrinogen stimulation of enzymatic activity of t-PA (clear bars) and S-NO-t-PA (hatched bars), compared in the coupled enzyme assay at concentrations of 0.1 μM and 1.0 μM of plasminogen.

Figure 7. Increases in intracellular platelet cyclic GMP, caused by S-NO-t-PA (Example 6.B.3).

Figure 8. Inhibition of platelet aggregation by S-NO-t-PA (Example 6.B.3).

8a: Illustrates inhibition of platelet aggregation by S-NO-t-PA (15 nM).

8b: Illustrates inhibition of platelet aggregation by S-NO-t-PA (150 nM).

8c: Illustrates inhibition of platelet aggregation by S-NO-t-PA (333 nM) synthesized from authentic EDRF.

Figure 9. Comparison of S-NO-t-PA-induced vasorelaxation caused by (a)t-PA (150 nM), (b) S-NO-t-PA (150 nM), and (c) S-NO-t-PA (150 nM) (Example 6.C.3).

Figure 10. Dose dependent relaxation of vascular smooth muscle and inhibition of platelet aggregation caused by S-nitroso-BSA (S-NO-BSA) (Example 7.A to 7.B.2).

Figure 11. Representative tracings of vessel relaxation and platelet inhibition caused by S-nitroso-BSA (S-NO-BSA).

11a: Illustrative tracings comparing the platelet inhibitory effects of (a) S-NO-BSA; (b) $NaNO_2$;

(c) BSA; (d) iodoacetamide-treated BSA exposed to NO generated from acidified $NaNO_2$ (Example 7.A).

11b: Illustrative tracings comparing the vasodilatory effects of (a) BSA (1.4 μM); (b) iodoacetamide-treated BSA treated with NO generated from acidified $NaNO_2$ as described in Figure 3a; (c) S-NO-BSA (1.4 μM) after platelets were pretreated with 1 μM methylene blue for ten minutes; (d) S-NO-BSA (1.4 μM) (Example 7.B.2).

Figure 12. Coronary blood flow in anesthetized dogs, following infusion of S-nitroso-BSA (Example 8).

Figure 13. Duration of increased coronary blood flow, following infusion of S-nitroso-BSA.

Figure 14. Coronary vasodilation, following infusion of S-nitroso-BSA.

Figure 15. Dose-dependent vasodilatory response caused by S-nitroso-cathepsin.

Figure 16. Tracings of dose-dependent inhibition of platelet aggregation caused by S-nitroso-LDL.

Figure 17. Representative tracings of vessel relaxation caused by S-nitroso-LDL.

Figure 18. Tracings of dose-dependent inhibition of platelet aggregation caused by S-nitroso-immunoglobulin.

Figure 19. Representative tracings of vessel relaxation caused by S-nitroso-immunoglobulin.

Figure 20. Concentration-dependent relaxation of airway smooth muscle caused by S-NO-BSA.

Figure 21. Nitrosylation of L-tyrosine.

21a: [^{15}N]-NMR spectrum.
21b: [^{1}H]-NMR spectrum.
21c: FTIR spectrum
21d: UV spectrum for 1.8 mM of tyrosine.
21e: UV spectrum for 34 mM of tyrosine.

Figure 22. Nitrosylation of L-phenylalanine [^{15}N]-NMR spectrum.

Figure 23. UV spectrum for nitrosylation of tryptophan.

23a: 5 minute reaction time.
23b: 10 minute reaction time.
23c: 15 minute reaction time.
23d: 30 minute reaction time.
23e: 60 minute reaction time.

Figure 24. [^{15}N] NMR for nitrosylated bovine serum albumin.

Figure 25. UV spectrum for time-dependent NO loading of BSA.

25a: 1 minute reaction time.
25b: 5 minute reaction time.
25c: 30 minute reaction time.

Figure 26. Nitrosylation of t-PA.

Figure 27. Vasodilatory effects of NO-loaded BSA.

Figure 28. S-nitrosylation of hemoglobin.

Figure 29. UV spectrum of hemoglobin incubated with S-nitroso-N-acetylcysteine.

Figure 30. Reaction of nitric oxide at the iron-binding site of hemoglobin.

Figure 31. UV spectrum of S-NO-hemoglobin (Hb). Spectra shows that oxygen binding to the heme site is largely unaffected at S-NO/Hb stoichiometries (0.05 and 0.37) that are lacking in smooth muscle contractile activity (see Figure 2). Higher ratios of S-NO/Hb (1.59) are associated with methemoglobin formation. Curves for oxy-Fe(II)-hemoglobin (dithionite-treated) and methemoglobin ($K_3Fe(CN)$-treated) are shown for comparison.

Figure 32. Reversal of hemoglobin (Hb)-induced contraction of aortic rings by S-nitrosylation. Hemoglobin is shown to constrict vessels in a dose-dependent manner (●). S-NO-Hb, with a stoichiometry of 0.1 S-NO\Hb, entirely prevents the constrictor response (O). Increasing the S-NO-Hb\Hb stoichiometry to 1 converts hemoglobin into a potent vasodilator (+).

Figure 33. Endogenous levels of S-NO-hemoglobin in rat artery, rat vein and human vein. Rat arterial blood was obtained by direct cardiac puncture, rat venous blood from jugular vein and human blood by venipuncture, according to approved protocols.

The invention is based on the discovery by the inventors that nitrosylation of proteins and amino acids provides a means by which protein and amino acid function may be selectively regulated, modified or enhanced.

A significant advantage of nitroso-proteins is that they deliver NO in its most biologically relevant, and non-toxic form. This is critical, because the pharmacological efficacy of NO depends upon the form in which it is delivered. This is particularly true in airways, where high levels of O_2 and O_2 reactive species predispose to rapid inactivation of the NO moiety. As demonstated by the inventors, nitroso-proteins deliver NO as the charged species, nitrosonium (NO^+) or nitroxyl (NO^-), and not the uncharged NO radical (NO•). This is important because the charged species behave in a very different manner from NO• with respect to chemical reactivity.

In contrast to NO•, nitrosonium and nitroxyl do not react with O_2 or O_2 species, and are also resistant to decomposition in the presence of redox metals. Consequently, administration of NO equivalents does not result in the generation of toxic by-products or the elimination of the active NO moiety. By delivering nitrosonium or nitroxyl, S-nitroso-proteins provide a means for achieving the smooth muscle relaxant and anti-platelet effects of NO, and at the same time, alleviate significant adverse effects previously associated with NO therapy.

The term "nitrosylation" refers to the addition of NO to a thiol group (SH), oxygen, carbon or nitrogen by chemical means. The source of NO may be endogenous NO or endothelium-derived relaxing factor, or other nitrosylating agents, such as nitroglycerin, nitroprusside, nitrosothiols, nitrous acid or any other related compound.

The term "regulated" means effective control of the activity of a protein or amino acid, in a selective manner so as to cause the protein or amino acid to exert a desired physiological effect.

The term "modified" means to effectively alter the activity of a protein or amino acid in a selective manner, so as to cause the protein or amino acid to exert a desired physiological effect.

The term "enhanced" means to alter effectively the activity of a protein or amino acid in a selective manner, so as to cause an increase or improvement in the activity of the protein or amino acid, or endow the protein or amino acid with additional capabilities.

The terms "mutant", "variant" or "fragment" refer to any structurally modified protein or polypeptide that retains the same physiological activity of interest, as the parent protein or polypeptide, whether to a greater or lesser extent. Other properties or advantages may be present in such mutant, variant or fragment compound, such as increased half-life or resistance to natural inhibitors of the parent protein. Mutation can refer, for example, to changes in one or more amino acids in the primary sequence. Variants can refer for example, to posttranslational modifications, such as glycosylation, conformational contraint, or the addition of lipid moieties. Fragments can refer to polypeptides that retain some, all or more of the desired physiological property of the parent protein while not having one or more amino acids or sequences thereof present in the parent protein.

The term "substantially homologous," refers to amino acid sequences of hemoglobins, modified hemoglobins and other heme-containing proteins, means that a particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between reference and

subject sequences. For purposes of the present invention, sequences having greater than 90 percent homology, equivalent biological activity, and equivalent expression characteristics are considered substantially homologous. For purposes of determining homology, truncation of the mature sequence should be disregarded. Sequences having lesser degrees of homology, comparable bioactivity, and equivalent expression characteristics are considered equivalents. Other embodiments include fusion protein products that exhibit similar oxygen carrying activities. Further embodiments include such proteins that are chemically synthesized as well as any proteins or fragments thereof that are substantially homologous.

The term "activity" refers to any action exerted by the protein or amino acid which results in a physiological effect.

The inventors have investigated the reaction of NO with protein thiols and have demonstrated that a variety of proteins of biological significance and relative abundance can be S-nitrosylated. S-nitrosylation of proteins endows these molecules with potent and long-lasting NO-like effects of vasodilation and platelet inhibition, mediated by guanylate cyclase activation, and also provides a means for achieving selective regulation of particular protein functions.

To develop the S-nitroso-protein compounds of the invention, certain thiol-containing proteins which are representative of various functional classes were nitrosylated. Such proteins include enzymes, such as tissue-type plasminogen activator (t-PA) and cathepsin B; transport proteins, such as lipoproteins, hemoglobin, and

serum albumin; and biologically protective proteins, such as immunoglobulins.

The data demonstrate that 1) NO can react with thiol groups in proteins to form S-nitrosothiols; 2) this reaction occurs under physiologic conditions; 3) these compounds are biologically active, exhibiting vasodilatory and antiplatelet properties that are independent of their method of synthesis; 4) the long chemical half-lives of S-nitroso-proteins vis-a-vis the half life of NO is reflected in their different relaxation kinetics: S-nitroso-proteins, through activation of guanylate cyclase, is fully consistent with that of other nitroso-compounds; although the possibility of other mechanisms by which S-NO-proteins can produce biologic effects cannot be excluded, such as the transfer of NO to another protein thiol, the function of which is thereby modulated. (Craven *et al., J. Biol. Chem.,* 253:8433 (1978);Katsuki *et al., J.Cyc. Nuc. Prot. Phos. Res.* 3:23 (1977); Osborne *et al., J. Clin. Invest.* 83:465 (1989)).

One aspect of the invention relates to nitrosylated enzyme compounds derived from nitrosylation of enzymatic proteins. A particular embodiment of this aspect relates to the compound, S-nitroso-t-PA (S-NO-t-PA), derived from the nitrosylation of tissue-type plasminogen activator (t-PA).

Acute occlusive events are precipitated by thrombogenic stimuli and alterations in flow dynamics within the vessel. Platelet activation, augmented local vasoconstriction, and recruitment of the coagulation system each plays a major role in the subsequent development of a thrombus (Marder *et al., New Engl. J. Med. 318*:1512,1520 (1988)). t-PA is one of the products secreted by blood vessel endothelium, which specifically counteracts these thrombogenic mechanisms. t-PA, a serine protease, converts

plasminogen to plasmin on fibrin and platelet thrombi, which in turn induces fibrinolysis and platelet disaggregation. Loscalzo *et al.*, *New Engl. J. Med., 319(14):925-931* (1989); Loscalzo *et al.*, *J. Clin. Invest.*, 79:1749-1755 (1987).

Attempts have been made to improve the thrombolytic efficacy and pharmacological properties of plasminogen activators, such as t-PA. In light of the role of platelets in clot formation and in reocclusive vascular events, one major focus has involved the use of ancillary antiplatelet therapy. Some success has been achieved with aspirin (ISIS-2 *Lancet*, *2*:349-360 (1988)), and other benefits are reported for several newer antiplatelet compounds (Gold, *H.K. New Engl. J. Med.*, *323*:1483-1485 (1990)). Attempts have also been made to improve the functional properties of the plasminogen activator itself through site-directed mutagenesis and synthesis of hybrid molecules and biochemical conjugates (Runge *et al.*, *Circulation*, *79*:217-224 (1989); Vaughan *et al.*, *Trends Cardivasc. Med.*, *Jan/Feb*:1050-1738 (1991)).

Motivated by the need for a plasminogen activator with improved thrombolytic efficacy and anti-thrombogenic properties, the inventors discovered that nitrosylation of t-PA creates a new molecule (S-NO-t-PA) which has improved thrombolytic capability, (e.g., the enzymatic activity of the enzyme is enhanced) as well as vasodilatory and platelet inhibitory effect. The inventors demonstrated that S-nitrosylation significantly enhances the bioactivity of t-PA, without impairing the catalytic efficiency or other domain-specific functional properties of the enzyme.

In particular, S-nitrosylation of t-PA at the free cysteine, cys 83, confers upon the enzyme potent antiplatelet and vasodilatory properties, without adversely affecting its

catalytic efficiency or the stimulation of this activity by fibrin(ogen). In addition, the S-nitrosothiol group does not appear to alter the specific binding of t-PA to fibrin(ogen) or the interaction of t-PA with its physiological serine protease inhibitor, PAI-1. The proteolytic activity, fibrin(ogen)-binding properties and regions for interaction with PAI-1 reside in several functional domains of the molecule that are linearly separate from the probable site of S-nitrosylation in the growth factor domain (cys 83). Thus, chemical modification of t-PA by NO does not markedly alter functional properties of t-PA residing in other domains. In addition, S-nitrosylation enhances the catalytic efficiency of t-PA against plasminogen, and increases its stimulation by fibrinogen.

NO is highly labile and undergoes rapid inactivation in the plasma and cellular milieu. This suggests that the reaction between No and the protein thiol provides a means of stabilizing NO in a form in which its bioactivity is preserved. Specifically, S-NO-t-PA is a stable molecule under physiologic conditions and, much like NO, is capable of vasodilation and platelet inhibition mediated by cyclic GMP. Stabilizing NO in this uniquely bioactive form creates a molecule with intrinsic vasodilatory, antiplatelet, and fibrinolytic properties, which enable it to counteract each of the major thrombogenic mechanisms.

Another embodiment of this aspect relates to the administration of S-NO-t-PA as a therapeutic agent to an animal for the treatment and prevention of thrombosis. Current thrombolytic strategies are based on the understanding of the endogenous mechanisms by which the endothelium protects against thrombogenic tendencies. In particular, platelet inhibition and nitrovasodilation are frequently used concomitant therapies with which to enhance

reperfusion by plasminogen activators as well as to prevent rethrombosis (Gold, H.K. *New Engl. J. Med.*, *323*:1483-1485 (1990); (Marder et *al.*, *New Engl. J. Med.*, *318*:1512-1520 (1988)).

Administration of S-NO-t-PA to a patient in need thereof provides a means for achieving "fibrin-selective" thrombolysis, while simultaneously attenuating the residual thrombogenicity resulting from simultaneous platelet activation and thrombin generation during thrombolysis. Furthermore, by virtue of its fibrin binding properties, S-NO-t-PA provides targeted delivery of the antiplatelet effects of NO to the site of greatest platelet activation, the actual fibrin-platelet thrombus. S-NO-t-PA has therapeutic application in the treatment or prevention of conditions which result from, or contribute to, thrombogenesis, such as atherothrombosis, myocardial infarction, pulmonary embolism or stroke.

In summary, S-NO-t-PA possesses unique properties that facilitate dispersal of blood clots and prevent further thrombogenesis. The discovery of this unique molecule provides new insight into the endogenous mechanism(s) by which the endothelium maintains vessel patency and offers a novel, and beneficial pharmacologic approach to the dissolution of thrombi.

Another embodiment of this aspect relates to compounds obtained by the nitrosylation of other thrombolytic agents, such as streptokinase, urokinase, or a complex containing one or more thrombolytic agents, such as streptokinase, urokinase, or t-PA. These compounds may also be administered to an animal, in the same manner as S-NO-t-PA for the treatment and prevention of thrombosis.

An additional aspect of this invention relates to compounds derived from the nitrosylation of other enzymes. One particular compound is S-NO-cathepsin, derived from the nitrosylation of cathepsin B, a lysosonml cysteine protease. The inventors have demonstrated that S-NO-cathepsin exerts a vasodilatory and platelet inhibitory effect. Thus, this compound may be administered as a therapeutic agent to an animal, to promote vasodilation and platelet inhibition, and to treat or prevent cardiovascular disorders.

Another aspect of the invention relates to nitroso-lipoprotein compounds derived from the nitrosylation of lipoproteins. Such lipoproteins include chylomicrons, chylomicron remnant particles, very low-density lipoprotein (VDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and high density lipoprotein (HDL) and lipoprotein (a). The inventors have demonstrated that S-nitroso-lipoproteins exert vasodilatory and platelet inhibitory effect. Thus, these compounds may be administered as a therapeutic agent, to an animal, to promote vasodilation and platelet inhibition, and to treat or prevent cardiovascular disorders.

An additional aspect of the invention involves the *in vivo* nitrosylation of lipoproteins as a means for regulating cellular uptake of lipoproteins. Consequently, nitrosylation provides a means for regulating lipid uptake, and treating or preventing disorders associated with hyperlipidemias, such as atherosclerosis.

Another aspect of the invention relates to the nitroso-immunoglobulin compounds derived from the nitrosylation of immunoglobulins. Such immunoglobulins may include IgG, IgM, IgA, IgD, or IgE. The inventors have demonstrated that these compounds exert vasodilatory and

platelet inhibitory effect. Thus, these compounds may be administered as therapeutic agents, to an animal, to promote vasodilation and platelet inhibition, and to treat or prevent cardiovascular disorders. The half lives of these compounds, in the order of one day, produce unique, long-lasting vasodilatory effects which are notably different from those of low molecular weight nitroso-compounds.

An additional and preferred aspect of the invention is nitrosylated blood substitutes, particularly nitrosylated cell-free blood substitutes such as those containing the compound nitroso-hemoglobin, derived from the nitrosylation of hemoglobin. This compound may be used as therapeutic agent to promote vasodilation and platelet inhibition, and to treat or prevent cardiovascular disorders. As demonstrated by the inventors, nitrosylation of hemoglobin increases its oxygen-binding capacity. Hemoglobin is a globular protein, which binds reversibly to blood oxygen through passive diffusion from entry of air into the lungs. Hemoglobin-oxygen binding greatly increases the capacity of the blood to transport oxygen to bodily tissues; thus, the binding affinity between hemoglobin and oxygen is a critical factor in determining the level of oxygen transport to the tissues. The thiol group on the hemoglobin molecule regulates the affinity of hemoglobin for oxygen. The inventors have demonstrated that some S-nitrosothiols, such as S-nitroso-proteins do not react with the iron-binding site of hemoglobin, as does NO, but instead, bind to the thiol group. Thus, methemoglobin formation is prevented and hemoglobin-oxygen binding is unimpaired.

Furthermore, the inventors have also demonstrated that nitrosylation of hemoglobin not only prevents impairment of binding, but actually increases hemoglobin-oxygen binding. Therefore, another embodiment of the invention involves the

administration of nitroso-hemoglobin or the *in vivo* nitrosylation of hemoglobin, to increase the oxygen-carrying capacity of the blood, and oxygen transport to bodily tissues. As a result, these compounds are also useful in the treatment of disorders which are associated with insufficient oxygen transport, or in clinical situations in which increased oxygen transport is needed. Examples of such clinical situations include, but are not limited to, hypoxic disorders resulting from pneumothorax, airway obstruction, paralysis or weakness of the respiratory muscles, inhibition of respiratory centers by drug or other agents, or other instances of decreased pulmonary ventilation. Additional clinical indications include impaired alveolar gas diffusion such as occurs in interstitial fibrosis, bronchiole constriction, pulmonary edema, pneumonia, hemorrhage, drowning, anemias, arteriovenous shunts, and carbon monoxide poisoning. In addition, nitroso-hemoglobin may also be used to modulate the delivery of carbon monoxide or nitric oxide (bound to hemoglobin) to bodily tissues. In addition, any thiol-containing heme proteins may be nitrosylated and used to enhance the oxygen-carrying capacity of the blood.

An additional aspect of the invention is the use of blood substitute compositions in accordance with the invention for maintaining and perfusing transplant organ or tissue materials such that they can be maintained for duration upon necessary to transport them or to culture expand them particularly when they are largely comprised of progenitor cells. Such profusion of organ and tissue maintenance compositions are in liquid form and constitute the compounds of the invention in physiologically acceptable carriers.

An additional aspect of the invention is nitroso-myoglobin, derived from the nitrosylation of myoglobin, a

protein which also transports oxygen. This compound may be used as a therapeutic agent to promote vasodilation and platelet inhibition, and to treat or prevent cardiovascular disorders.

Another aspect of the invention relates to a method for using S-nitroso-proteins as a means for providing targeted delivery of NO. The term "targeted delivery" means that NO is purposefully transported and delivered to a specific and intended bodily site. In the same manner as S-NO-t-PA, SNO-immunoglobulin can be modified, by cationic modification of the heavy chain, to provide targeted delivery of NO to the basement membrane of the glomerulus in the kidney. Successful delivery of four NO molecules per immunoglobulin have been directed to the kidney basement membrane in this matter. Targeted delivery of NO provides a means for achieving site-specific smooth muscle relaxation, or other NO-mediated effects. In addition, delivery may be for the purpose of nitrosylation of various molecules present in the body. For example, S-nitroso-proteins would deliver NO, and thus nitrosylate hemoglobin or myoglobin in order to increase oxygen binding.

Another aspect of the invention relates to the administration of nitroso-albumin as a therapeutic agent to promote platelet inhibition, or to cause relaxation of airway smooth muscle. The inventors have demonstrated that S-nitroso-BSA exerts a platelet inhibitory effect, and also promotes long-acting vasodilatory effect, which can be distinguished from that of NO or the low molecular weight thiols.

The inventors have also demonstrated that S-nitroso-BSA relaxes human airway smooth muscle. As discussed above, by delivering NO in the form of charged NO

equivalents, such as nitrosonium, S-nitroso-proteins cause airway relaxation, and also eliminate the adverse effects which occur with administration of other NO species. Thus, S-nitroso-albumin may be administered for the treatment or prevention of respiratory disorders including all subsets of obstructive lung disease, such as emphysema, asthma, bronchitis, fibrosis, excessive mucous secretion and lung disorders resulting from post surgical complications. In addition these compounds may be used as antioxidants, and thus, in the treatment of diseases such as acute respiratory distress syndrome (ARDS).

Another aspect of the invention relates to a method for nitrosylation of those proteins which lack free thiols. The method involves thiolating the protein by chemical means, such as homocysteine thiolactone (Kendall, *BBA*, *257*:83 (1972)), followed by nitrosylation in the same manner as the compounds discussed above. Recombinant DNA methods may also be used to add or substitute cysteine residues on a protein.

Another aspect of the invention relates to a method for nitrosylation of those proteins in which the thiol is blocked by a methyl group. The method involves selective de-methylation of the protein by chemical means, such as reacting with methyl transferase, followed by nitrosylation in the same manner as the compounds discussed above.

Another aspect of the invention involves the use of S-nitroso-protein compounds to relax non-vascular smooth muscle. Types of smooth muscle include, but are not limited to, bronchial, tracheal, uterine, fallopian tube, bladder, urethral, urethral, corpus cavernosal, esophageal, duodenal, ileum, colon, Sphincter of Oddi, pancreatic, or common bile duct.

Another aspect of the invention involves the *in vivo* nitrosylation of protein thiols, by administration of a nitrosylating agent as a pharmaceutical composition. *In vivo* nitrosylation provides a means for achieving any of the physiological effects discussed above, or for regulation of additional protein functions.

Proteins and amino acids possess other sites, In addition to thiol groups, in addition to thiol groups, which can be nitrosylated. For example, such sites may include, but are not limited to, oxygen, nitrogen, and carbon. Thus, as noted above the invention also relates to the nitrosylation of additional sites, such as oxygen, nitrogen, and carbon which are present on proteins and amino acids, as a means for achieving any of the physiological effects discussed above, or for regulation of additional protein or amino acid functions. The inventors have shown that aromatic amino acids, such as tyrosine, phenylalanine and tryptophan can be nitrosylated at the hydroxyl, and amino groups, as well as on the aromatic ring, upon exposure to nitrosylating agents such as $NaNO_2$, NOCl, N_2O_3, N_2O_4 and NO^+.

The hydroxyl group of tyrosine also plays a central role in a variety of cell regulatory functions. For example, phosphorylation of tyrosine is a critical cell regulatory event. In addition, serine residues also provide phosphorylation sites. Thus, the invention provides for the nitrosylation of amino acids such as tyrosine and serine, to regulate cellular process such as, but not limited to, phosphorylation.

Another embodiment of the invention relates to the use of O-nitrosylation of tyrosine residues on bovine serum albumin as a method for achieving smooth muscle relaxation

and platelet inhibition. Other amino acids, such as serine and threonine may also be nitrosylated in the same manner.

The ability to bind NO to a variety of different sites on an amino acid or protein provides a greater concentration of NO, and thus may enhance regulation of protein function, as well as other NO-mediated effects such as smooth muscle relaxation and platelet inhibition. Thus, another embodiment of the invention relates to the use of amino acids and proteins which contain numerous NO molecules, to regulate protein or amino acid function and to effect smooth muscle relaxation and platelet inhibition. Additional therapeutic uses of these compounds include the treatment or prevention of such disorders as heart failure, myocardial infarction, shock, renal failure, hepatorenal syndrome, post-coronary bypass, gastrointestinal disease, vasospasm of any organ bed, stroke or other neurological disease, and cancer.

Another embodiment of the invention relates to a method for using these nitrosylated proteins and amino acids as a means for providing targeted delivery of NO to specific and intended bodily sites. These compounds have the capacity to deliver charged NO equivalents. For example, alkyl nitrites having the formula X-CONO and containing a beta-election withdrawing group would be able to deliver these charged NO equivalents.

Another aspect of the invention relates to the administration of a pharmaceutical composition comprised of any S-nitroso-protein, to inhibit platelet function, cause vasodilation, relax smooth muscle, deliver nitric oxide to specific targeted bodily sites, or for the treatment or prevention of cardiovascular or respiratory disorders.

An additional application of the present invention relates to the nitrosylation of additional compounds such as peptides, neurotransmitters, pharmacologic agents and other chemical compounds, as a therapeutic modality. For example, nitrosylation of dopamine, a neurotransmitter improves the cardiac profile of the drug, by enhancing afterload reduction and scavenging free radicals, while simultaneously inhibiting platelets and preserving renal blood flow. Nitrosylation of epinephrine and related sympathomimetic drugs alters the half-life of the drug and affects its β-agonist selectivity.

The nitrosylated proteins and amino acids of the present invention, or the nitrosylating agents may be administered by any means that effect thrombolysis, vasodilation, platelet inhibition, relaxation of non-vascular smooth muscle, other modification of protein functions or treatment or prevention of cardiovascular disorders, or any other disorder resulting from the particular activity of a protein or amino acid. For example, administration may be by intravenous, intraarterial, intramuscular, subcutaneous, intraperitoneal, rectal, oral, transdermal or buccal routes.

According to the present invention, a "therapeutically effective amount" of therapeutic composition is one which is sufficient to achieve a desired biological effect. Generally, the dosage needed to provide an effective amount of the composition, which can be adjusted by one of ordinary skill in the art, will vary, depending on the age, health, condition, sex, weight, and extent of disease, of the recipient. In addition, the dosage may also depend upon the frequency of treatment, and the nature of the effect desired.

Compositions within the scope of this invention include all compositions wherein the S-nitroso-protein or the nitrosylating agent is contained in an amount effective to

achieve its intended purpose. While individuals needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosage forms contain 1 to 100 mmol/kg of the S-nitroso-protein. The dosage range for the nitrosylating agent would depend upon the particular agent utilized, and would be able to be determined by one of skill in the art.

In addition to the pharmacologically active compounds, the new pharmaceutical preparations may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain preferably, about 0.01 to 5 percent, preferably from about 0.1 to 0.5 percent of active compound(s), together with the excipient.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as sugars, for example lactose or sucrose, mannitol or sorbitol,

cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methylcellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethylstarch, cross-linked polyvinyl pyrrolidone, agar, or algenic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropymethyl-cellulose phthalate, are used. Dye stuffs or pigments may be added to the tablets or dragee coatings, for example, for identification or in order to characterize combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and,

optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, or liquid paraffin. In addition, stabilizers may be added.

Possible pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of the active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

Preferred fatty acids generally contain from 4-36 carbon atoms and preferably contains at least 12 carbon atoms, most preferably 12 to 22. In some cases this carbon chain is fully saturated and unbranched, while others contain one or more double bonds. They can have saturated, unsaturated, branched or straight chain hydrocarbon chains.

A few contain 3-carbon rings or hydroxyl groups. The compounds are not surface active. They are poorly soluble in water and the longer the acid chain and the fewer the double bonds, the lower the solubility in water. The carboxylic acid group is polar and ionized at neutral pH. This accounts for the slight solubility of short-chain acids in water.

Examples of such acids are those ranging from C_{16} to C_{22} with up to three unsaturated bonds (also branching). Examples of saturated straight chain acids are n-dodecanoic acid, n-tetradecanoic acid, n-hexadecanoic acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, montanic acid and melissic acid. Also useful are unsaturated monoolefinic straight chain monocarboxylic acids. Examples of these are oleic acid, gadoleic acid and erucic acid. Also useful are unsaturated (polyolefinic) straight chain monocaboxyic acids. Examples of these are linoleic acid, ricinoleic acid, linolenic acid, arachidonic acid and behenolic acid.

The composition or preparation of the invention can further include a surfactant, or a mixture of two or more surfactants. A surfactant is an amphiphilic molecule consisting of a hydrophobic tail and a hydrophilic head. These molecules possess distinct regions of both hydrophilic and hydrophobic character. The hydrophobic tail can be a hydrocarbon or fluorocarbon chain of 8 to 18 carbon atoms. They are long chain molecules such as, for example, soaps or detergents. Surfactants accumulate at the hydrophilic/hydrophobic (water/oil) interface and lower the surface tension. Surface active agents or surfactants are long chain molecules, such as soaps and detergents, which accumulate at the hydrophilic/hydrophobic(water/oil) interface and lower the surface tension at the interface. One effect of a

reduced surface tension is the stabilization of the emulsions. This is because molecules with both polar and non-polar groups become oriented such that the hydrocarbon tail embeds itself into the hydrophobic phase and the hydrophilic head protrudes into the hydrophilic phase. Where the hydrophobic composition or other component of the preparation includes a surface-active agent, such as a surfactant, it is usually present in amounts of about 0.05% to 50.0% weight/weight of the hydrophobic composition with a preferred range of 1.0% to 3.0% (w/w). Preferred surfactants include, for example, the Tween(polyoxyethylene sorbate) family of surfactants(ICI, Wilmington DE), the Span(sorbitan long chain carboxylic acid esters) family of surfactants(ICI), the Pluronic(ethylene or propylene oxide block copolymers) family of surfactants(BASF, Parsippany NJ), the Labrasol, Labrafil and Labrafac(each polyglycolyzed glycerides) families of surfactants(Gappe Fosse, St. Priest, France), sorbitan esters of oleate, stearate, laurate or other long chain carboxylic acids, poloxamers (polyethylene-polypropylene glycol block copolymers), other sorbitan or sucrose long chain carboxylic acid esters, mono and diglycerides, PEG derivatives of caprylic/capric triglycerides and mixtures thereof.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

Example 1

Synthesis of S-Nitroso-t-PA

A. Nitrosylation of t-PA

1. Materials

t-PA was kindly provided by Genentech, Inc. San Francisco, CA. Reactivated purified plasminogen activator

inhibitor-1 (PAI-1) and a panel of six murine anti-t-PA monoclonal antibodies were kindly provided by Dr. Douglas E. Vaughan. Horse-Radish Peroxidase linked-sheep antimurine antibodies were purchased from Amersham Corp., Arlington, II. Sodium nitrite was purchased from Fisher Scientific, Fairlawn, NJ. H-D-isoleucyl-L-prolyl-L-arginyl-p-nitroanilide (S2288) and H-D-valyl-L-leucyl-L-lysyl-p-nitroanilide (S2251) were purchased from Kabi Vitrum, Stockholm, Sweden. Human fibrinogen purified of plasminogen and von Willebrand factor, was obtained from Enzyme Research Laboratories, South Bend, IN. Epinephrine, ADP and iodoacetamide were purchased from Sigma Chemical Co., St. Louis, MO. Bovine thrombin was obtained from ICN, ImmunoBiologicals (Lisle, IL). Radioimmunoassay kits for the determination of cGMP were purchased from New England Nuclear, Boston, MA.

2. Plasminogen Preparation

Glu-plasminogen was purified from fresh frozen plasma thawed at 37°C using a modification of the method of Deutsch and Mertz (Deutsch *et al.*, *Science*, 170:1095-1096 (1970), herein incorporated by reference). Plasma was passed over a lysine-Sepharose column and the column washed with 0.3 M sodium phosphate, pH 7.4, 3 mM EDTA. Plasminogen was eluted from the column with 0.2 M epsilon-aminocaproic acid, 3 mM EDTA, pH 7.4. Contaminant plasmin was removed by passing the eluted column over benzamidine sepharose 2B. The plasminogen obtained was subsequently dialyzed before use against 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl.

3. Thiol Derivatization

The free thiol of t-PA was carboxyamidated by exposure of the enzyme to a 10-fold excess of iodoacetamide in the dark for one hour at 37°C in 10 mM Tris, pH 7.4, 0.15 M NaCl (TBS). t-PA was then dialyzed extensively against 10 mM HCl in order to remove excess iodoacetamide.

4. Microcarrier Endothelial Cell Culture

Endothelial cells were isolated from bovine aorta by established techniques (Schwartz, S.M. *In Vitro* 14:966-980 (1978), herein incorporated by reference) and cultured on a microcarrier system of negatively charged spherical plastic beads (Biosilon), according to the method of Davies and colleagues (Davies *et al.*, *J. Cell Biol.* 101:871-879 (1985), herein incorporated by reference).

5. Nitrosylation

t-PA was first dialyzed against a large excess of 10 mM HCl for 24 hours to remove excess L-arginine used to solubilize the protein. t-PA was then exposed to NO_x generated from equimolar $NaNO_2$ in *0.5 N* HCl (acidified $NaNO_2$) or in control experiments, to *0.5 N* HCl alone, for 30 minutes at

37°C. Solutions were titrated to pH 7.4 with equal volumes of 1.0 N NaOH and Tris Buffered Saline (TBS), pH 7.4, 0.05 M L-arginine. Dilutions were then made as necessary in TBS.

For comparative purposes, and to illustrate the potential biological relevance of S-NO-t-PA, this compound was synthesized with authentic EDRF in selected experiments. In this method, t-PA was incubated with bovine aortic endothelial cells stimulated by exposure to high shear forces to secrete EDRF, as we have previously described (Stamler *et al., Cir. Res.*, 65:789 (1989), herein incorporated by reference). Owing to the stability of the S-NO bond in S-NO-t-PA under physiologic conditions ($t_{1/2}$ > 24 hours in TBS, pH 7.4, 20°C), samples were stored at pH 7.4 on ice throughout the course of the experiments.

S-NO-t-PA has also been synthesized by exposure of t-PA to NO gas bubbled into buffered (TBS) solution of enzyme. This further illustrates the potential for S-nitrosylation, by exposure of proteins to a variety of oxides of nitrogen including NOCl, N_2O_3, N_2O_4, and other nitroso-equivalents.

B. Confirmation of S-NO bond

1. Methods

The formation and stability of the S-NO bond were confirmed by several published analytical methods.

In the first, NO displaced from S-nitrosothiol groups with Hg^{2+} was assayed by diazotization of sulfanilamide and subsequent coupling with the chromophore N-(1-naphthyl-)ethylenediamine (Saville, B., *Analyst*, 83:670-672 (1958), herein incorporated by reference).

In the second, the characteristic absorption spectrum of S-nitrosothiols in the range of 320 nm - 360 nm was

detected (Stamler *et al., Proc. Natl. Acad. Sci. USA,* CITE!!! (1991); Oac et al., *Org. Prep. Proc. Int.* 15(3):165-169 (1983)).

In the third, [^{15}N] NMR was used. Measurements of RS-NOs were made according to the method of Bonnett and colleagues (Bonnett *et al., JCS Perkins Trans.* 1:2261-2264 (1975), herein incorporated by reference). [^{15}N]NMR spectra were recorded with a Brucker 500 MHZ spectrometer, Billerica, MA. Deuterium lock was effected with $[D]_2O$ and the spectra referenced to an [^{15}N] natural abundance spectrum of a saturated solution of $NaNO_2$ at 587 ppm. Spectra were recorded at 50.68 MHZ and the nine transients of 16k data points collected with a 30° pulse width and a 10-second relaxation delay. Data were multiplied by a 2-Hz exponential line broadening factor before Fourier transformation.

Confirmation of the above chemical evidence for protein S-nitrosothiol synthesis was obtained by UV, NMR and IR spectroscopy. Previous characterization of S-nitrosothiols, revealed that they possess UV absorption maxima at 320 - 360 nm, chemical shifts of approximately 750 ppm relative to nitrite (Bonnett *et al., JCS Perkins Trans.* 1:2261-2264 (1975)), and IR stretches at approximately 1160 cm^{-1} and 1170^{-1} cm. (Loscalzo *et al., JPET,* 249:726-729 (1989)).

2. Results

In accordance with these observations, S-NO-t-PA exhibited an absorption maximum at 322 nm (Figure 1a), and a chemical shift at 751 ppm (relative to nitrite) (Figure 1b); elimination of the chemical shift was achieved by sample treatment with excess $HgCl_2$. In addition, the presence of two absorption bands at 1153 cm^{-1} and 1167 cm^{-1}, is entirely consistent with the formation of an S-nitrosothiol bond (*Myers et al., Nature, 345*:161-163 (1990); Oac *et al., Org.*

Prep. Proc. Int. 15(3):165-169 (1983); Bonnett *el al., JCS Perkins Trans. 1*:2261-2264 (1975). The quantification of NO (Protein-NO + free NO_x in the Saville reaction, and the NMR results demonstrating a single chemical shift, reveal that all NO bound to the protein exists in the form of an S-nitrosothiol.

Figure 2 illustrates the time-dependent formation of S-NO-t-PA. Aliquots of the solution containing $NaNO_2$ were removed sequentially for determination of S-NO bond formation (Schwartz, S.M. *In Vitro* 14:966-980 (1978)). Results are expressed as mean ± S.D. (n = 3). By 30 minutes of exposure to acidified $NaNO_2$, S-nitrosylation is essentially complete; the stoichiometry of -S-NO/t-PA (mol/mol) is 0.0 ± 0.1 (n = 3) at the completion of the reaction as determined by the method of Saville (Saville, B. *Analyst* 8&3:670-672 (1958)). Carboxyamidation of t-PA's free thiol with iodoacetamide completely prevents S-nitrosothiol formation as determined by this chemical method (Saville, B. *Analyst* 83:670-672 (1958)).

Figure 2 also illustrates the effect of acid treatment on the amidolytic activity of t-PA. At different intervals, aliquots of the enzyme exposed to 0.5 N HCl alone were neutralized, and amidolytic activity was assayed using the chromogenic substrate S2288. Results are expressed as mean ± S.D. (n = 3), relative to t-PA not treated with 0.5 N HCl. At 30 minutes, the duration of exposure subsequently used for S-nitrosodiiol synthesis, the enzymatic activity of t-PA is largely preserved. Quantification of S-NO-t-PA synthesis with authentic EDRF was similarly determined by the method of Saville (Saville, B. *Analyst* 83:670-672 (1958)).

Example 2

Synthesis of S-Nitroso-BSA

A. Nitrosylation

In the first method, nitrosylation of BSA was accomplished by incubating BSA (200 mg/ml with NO generated from equimolar $NaNO_2$ in 0.5N HCl (acidified $NaNO_2$) for thirty minutes at room temperature. Solutions were titrated to pH 7.4 with equal volumes of 1.0 N NaOH and Tris Buffered Saline (TBS), pH 7.4, 0.05 M L-arginine. Dilutions were then made as necessary in TBS.

In the second method, nitrosylation was achieved in helium-deoxygenated solutions of 0.1 M sodium phosphate (pH 7.4) by exposing the protein solution in dialysis tubing to authentic NO gas bubbled into the dialysate for fifteen minutes. The proteins were then dialyzed against a large excess of 0.01 M phosphate buffer at pH 7.4 to remove excess oxides of nitrogen.

In the third method, proteins were incubated with bovine aortic endothelial cells stimulated by exposure to high shear forces to secrete EDRF, as in Example 1(A). As a corollary of this method, proteins were also incubated directly with NO synthase purified from bovine cerebellum (Bredt *et al., Proc. et al., Proc. Natl. Acad. Sci. USA* 87:682 (1990), herein incorporated by reference) in the presence of the substrate Larginine and cofactors required for enzyme activity (Ca^{-}', calmodulin, and NADPH).

B. Confirmation of S-nitroso-protein formation

The formation and stability of the S-nitroso-protein was confirmed by several published analytical methods. NO displaced from S-nitroso thiol groups with Hg^{2+} was assayed by diazotization of sulfanilamide and subsequent coupling with the chromophore N-(1-naphthyl-ethylenediamine (Mellion *et*

al., Mol. Pharmacol. 23:653 (1983); Saville, B. *Analyst* 83:670 (1958)). The stoichiometries of S-NO-BSA determined by these chemical methods is shown in Table 1.

Confirmatory evidence for S-nitrosothiol bond formation in proteins was obtained by spectrophotometry; S-nitrosothiols possess dual absorption maxima at 320-360 nm and at approximately 550 nm (Oae *et al., Organic Prep. and Proc. Int. 15:165* (1983); Ignarro *et al., J. Pharmacol. Exp. Ther.* 218:739 (198 1); Mellion *et al., Mol. Pharmacol. 23:653* (1983); Loscalzo, *J., Clin. Invest.* 76:966 (1985)).

As one additional, more specific measure of protein S-nitrosylation, [^{15}N]-NMR spectroscopy was used. BSA was S-nitrosylated with Na[^{15}N]O_2, and the [^{15}N]-NMR spectrum of the resulting species recorded in Figure 3. Figure 3 demonstrates the [^{15}N]-NMR Spectrum of [^{15}N]-labeled S-nitroso-BSA. The chemical shift for S-nitroso-BSA was 703.97, which falls into the same range as other S-nitrosothiols (e.g., S-nitroso-L-cysteine) prepared under similar conditions (Bennett *et al., J. Chem. Soc. Perkins Trans. 1:2261* (1975)). The spectrum was recorded at 50.68 MHZ and the nine transients of 16K data points were collected with a 30° pulse width and a 2.5-sec relaxation delay. Data were multiplied by a 2-Hz exponential line broadening factor before Fourier transformation. The region of 590 to 810 ppm is displayed.

Example 3

Synthesis of S-Nitroso-Cathepsin B

Nitrosylation of cathepsin, and determination of S-nitrosothiol formation, was accomplished according to the methods described in Example 2. The stoichiometry of S-nitrosothiol/protein molecules for cathepsin is shown in Table 1.

Example 4

Synthesis of S-Nitroso-Lipoprotein

Synthesis was accomplished by nitrosylating purified low-density lipoprotein (LDL) according to the methods described in Example 2. Confirmation of S-nitroso-protein formation was verified according to the methods of Example 2. The stoichiometry of S-nitrosothiol/protein molecules for LDL is shown in Table 1.

Example 5

Synthesis of S-Nitroso-Immunoglobulin

Synthesis was accomplished by nitrosylating purified gamma globulin (Sigma) according to the methods described in Example 2. Confirmation of S-nitroso-protein formation was verified according to the methods of Example 2. The stoichiometry of S-nitrosothiol/protein molecules for immunoglobulin is shown in Table 1.

Table 1

S-Nitroso-Protein Synthesis

-S-NO/protein (mol/mol)	
Bovine Serum Albumin	0.85 ± 0.04
t-PA	0.88 ± 0.06
Cathepsin B	0.90 ± 0.02
Human plasma	0.87 ± 0.02
Immunoglobulin	0.35 ± 0.01
Lipoprotein (LDL)	1.80

The stoichiometries for the individual -S-NO/protein molar ratios are given in the table and represent the mean ± SEM of 3 to 6 determinations.

Example 6

Thrombolytic Anti-Platelet & Vasodilatory Effect of S-NO-t-PA

A. Thrombolysis

1. Fibrinogen Binding

The binding of t-PA and S-NO-t-PA to fibrinogen was measured using polystyrene microliter wells (flat-bottom, high binding 96-well EIA plates, cat. #3590, Costar, Cambridge, MA). Wells were coated with fibrinogen (0.08 $\mu g/\mu l$) and the remaining binding sites with 2% bovine serum albumin. Quantification of t-PA binding was determined using a Horse-Radish Peroxidase linked sheep antimurine antibody in a colorimetric assay in the presence of O-phenylenediamine, 0.014% H_2O_2. Color change was measured spectrophotometrically with a Dynatech MR500 Card Reader (Dynatech, Chantilly, VA) at 490 nm.

Binding of t-PA is reversible and specific, and saturates at 1500-3000 nM; at saturation, 18 ng of t-PA are bound per well (0105 moles t-PA per mole of fibrinogen) with an estimated K_D in the range of 15-650 nM. Binding of t-PA and S-NO-t-PA was quantified by ELISA over the concentration range of 150-1500 nM using a mixture containing six murine monoclonal anti-t-PA antibodies.

Comparison of t-PA and S-NO-t-PA. The binding of t-PA to fibrin(ogen) accounts for the relative "fibrin--specificity" of the enzyme as compared to certain other plasminogen activators (Loscalzo *et al., New Engl. J. Med.* 319(14):925-931 (1989); Vaughan *et al., Trends Cardiovasc. Med. Jan/Feb:1050-1738* (1991)). The effect of S--nitrosylation on this functional property of the enzyme was therefore assessed. The binding isotherms for t-PA and its S-nitrosylated derivatives were not significantly different from each other by two-way ANOVA. Therefore, these data were subjected to a single best-curve-fit binding isotherm (Figure

4). From a Scatchard analysis, the estimated apparent D_n of S-NO-t-PA for surface-bound fibrinogen is 450 nm, which falls well within the reported range for t-PA (Ranby, M., *Biochim. Biophysics Acta* 704:461-469 (1982)).

2. Measurement of Enzymatic Activity

The amidolytic activities of t-PA and its S-nitrosylated derivative were measured using the relatively specific chromogenic substrate, S2288. Substrate hydrolysis was measured spectrophotometrically at 405 nm with a Gilford Response UV/Vis Spectrophotometer (CIBA-Corning, Oberlin, OH). Activity was measured at 25°C in TBS using substrate concentrations varying from 0.1-2.0 mM and t-PA at a concentration of 100 nM. Kinetic parameters were determined from initial rates by double reciprocal plot analysis. The assessment of inhibition of t-PA and S-NO-t-PA enzymatic activity by PAI-1 was made at an enzyme concentration of 10 nM and a molar ratio of t-PA to active PAI-1 of 1.0. The degree of inhibition was determined relative to the initial rates in the absence of the inhibitor.

In the coupled enzyme assay, t-PA and S-NO-t-PA activities were assayed using the native substrate S2251. In selected experiments, fibrinogen stimulation of enzymatic activity was assessed at a fibrinogen concentration of 1 mg/ml. Substrate hydrolysis was measured spechtrophotometrically with a Dynatech MR 5000 Card Reader (Dynatech, Chantilly, VA) in TBS, pH 7.4, at 25°C. Initial reaction velocity was determined from the slope of the plot of absorbance (at 405 nm)/time vs. time (Ranby, M., *Biochem. Biophysic Acta* 704:461-469 (1982)). Using glu-plasminogen concentrations ranging from 0.1-10 μM at an S2251 concentration of 0.8 mM. Kinetic parameters were determined from initial rates by double reciprocal plot analysis.

Comparison of t-PA and S-NO-t-PA. The amidolytic activity of t-PA and S-NO-t-PA were first compared against the chromogenic substrate S2288. From a double reciprocal plot analysis it is evident that the kinetic parameters (K_m and V_{max}) and the catalytic efficiency of (K_{cat}/K_m) these molecules are essentially identical, as shown in Figure 5a. The values of these kinetic constants are provided in Table 2.

The effect of S-nitrosylation on the ability of t-PA to activate its physiologic substrate, plasminogen, was assessed in the coupled enzyme assay in the presence and absence of fibririogen. As seen in the Lineweaver-Burke plot (Figure 5b) and from the derived kinetic parameters (Table 2), S-NO-t-PA has a K_m for substrate similar to "wild type" t-PA. However, S-NO-t-PA has a slightly, but significantly, greater V_{max} yielding a catalytic efficiency that is 23% greater than that of native t-PA.

3. Discussion

Both fibrin and fibrinogen increase the rate of activation of plasminogen by t-PA. The enhanced enzymatic activity of t-PA is the result of its ability to bind directly fibrin(ogen), which brings about a conformational change either in t-PA or plasminogen that promotes the interaction of t-PA with its substrate (Loscalzo *et al.*, *New Engl. J. Med., 319(14):925-931* (1989)).

The consequences of S-nitrosylation on these important functional properties of t-PA were therefore studied in a comparative analysis with t-PA in the coupled enzyme assay. The results, summarized in Figure 6, indicate that S-NO-t-PA binds to fibrinogen; that as a result of this binding its enzymatic activity is enhanced; and that in the presence of physiologic (1 μM) plasminogen concentrations, the degree of

stimulation is equivalent to that of "wild type" t-PA. At lower plasminogen concentrations (0.1 μM), fibrinogen stimulation of S-NO-t-PA was 3.5-fold greater than t-PA (1 μM) ($p < 0.05$). Absolute rates of plasminogen activation were again slightly greater for S-NO-t-PA (vida *supra*).

t-PA is rapidly inhibited by its cognate plasma serpin, PAI-1 (Loscalzo *et al.*, *New Engl. J. Med.*, 319(14):925-931 (1989); Vaughan *et al.*, *Trends Cardiovasc. Med.*, *Jan/Feb:*1050-1738 (1991)). By serving as a pseudosubstrate, PAI-1 reacts stoichiometrically with t-PA to form an inactive complex. PAI-1 was equally effective at inhibiting the hydrolytic activity of t-PA and S-NO-t-PA in the direct chromogenic assay with S2288 (n = 3; P NS). Thus, S-nitrosylation of t-PA does not appear to alter its interaction with PAI-1.

B. Platelet Inhibition

1. Preparation of Platelets

Venous blood, anticoagulated with 1- mM trisodium citrate, was obtained from volunteers who had not consumed acetylsalicylic acid for at least ten days. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 g for ten minutes at 25°C. Platelet counts were determined with a Coulter counter (model ZM; Coulter Electronics, Hialeah, FL).

2. Platelet Gel-Filtration and Aggregation

Platelets were gel-filtered on a 4 x 10 cm column of Sepharose 2B in Tyrode's HEPES buffer as described previously (Hawiger *el al.*, *Nature*, 2831:195-198(1980), herein incorporated by reference). Platelets were typically suspended at a concentration of 1.5 x 10^8/ml and were used within 30 minutes of preparation. Platelet aggregation was monitored using a standard nephelometric technique (Born, *et al.*, *J. Physiol.*, *168:178-195* (1963), herein incorporated by

reference), in which 0.3-ml aliquots of gel-filtered platelets were incubated at 37°C and stirred at 1000 rpm in a PAP-4 aggregometer (Biodata, Hatboro, PA). Gel-filtered platelets were preincubated with t-PA or S-NO-t-PA for up to 45 minutes and aggregations induced with 5 μM ADP or 0.025 U/ml thrombin.

Aggregations were quantified by measuring the maximal rate or extent of light transmittance and expressed as a normalized value relative to control aggregations.

3. Cyclic Nucleotide Assays

The antiplatelet actions of S-nitrosothiols are mediated by cyclic GMP. Measurements of cGMP were performed by radioimmunoassay. Gel-filtered platelets were pre-incubated for 180 seconds with S-NO-t-PA (9 μM), and related controls. Reactions were terminated by the addition of 10% trichloracetic acid. Acetylation of samples with acetic anhydride was used to increase the sensitivity of the assay.

S-NO-t-PA incubated with platelets for 180 seconds, induced an 85% increase in intracellular cyclic GMP above basal levels in the presence of t-PA ($p < 0.01$). The elevation in intracellular platelet cGMP induced by S-NO-t-PA was entirely prevented by preincubation of platelets with the guanylate cyclase inhibitor methylene blue (10μM for ten minutes ($n = 3$) (Figure 7).

4. Discussion

The effects of S-NO-t-PA were studied in a gel-filtered platelet preparation. In these experiments, NO, generated for $NaNO_2$ had no significant effect on the extent of platelet aggregation (tracing not shown). Mean results for inhibition by S-NO-t-PA are presented in Table 4.

Figure 8 illustrates platelet inhibition induced by S-NO-t-PA (333 nm) synthesized with EDRF. In these experiments, t-PA was exposed to endothelial cells stimulated to secrete EDRF for 15 minutes after which the formation for S-NO-t-PA was verified by the Saville method (Saville, B. *Analyst*, 83:670-672 (1958)). S-NO-t-PA was then preincubated with platelets for ten minutes prior to induction of aggregation with 5 μM ADP. In the absence of t-PA, effluent from endothelial cells stimulated to secrete EDRF had no significant effect on platelet aggregation. S-NO-t-PA inhibited platelet aggregation to 5 μM ADP in a dose-dependent manner, with 50 ± 16% (mean ± S.D.) inhibition in rate and extent of aggregation observed at 1.4 μM S-NO-t-PA ($n = 4$; $p < 0.001$ vs. control). Inhibition of platelet aggregation induced by ADP (5 μM) or thrombin (0.024 U/ml) was demonstrable at concentrations of S-NO-t-PA in the pharmacologic range of 15-150 nM, as shown in the illustrative tracings of Figure 8(a) and (b) and in Table 4. In further support of the potential biological relevance for RS-NOs, and the comparable bioactivity of S-NO-t-PA irrespective of its method of synthesis, inhibition of platelet aggregation by S-NO-t-PA (333 nM) synthesized with authentic EDRF is illustrated in Figure 8(c).

C. Vasodilation

1. Preparation of Blood Vessels

New Zealand White female rabbits weighing 3-4 kg were anesthetized with 30 mg/kg IV sodium pentobarbital. Descending thoracic aortae were isolated and placed immediately in a cold physiologic salt solution (Kreb's) (mM): NaCl, 118; CK1, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 12.5; and D-glucose, 11.0. The vessels were cleaned of adherent connective tissue, and the endothelium removed by gentle rubbing with a cotton tipped applicator inserted into the lumen, after which the vessel was cut into 5 mm rings.

The rings were mounted on stirrups and connected to transducers (model FTO3C Grass Instruments, Quincy, MA) by which changes in isometric tension were recorded.

2. Bioassay

Samples were added to a standard bioassay in which vessel rings were suspended in glass chambers containing seven ml of oxygenated Kreb's buffer (Cook *et al., Am. J. Physiol.* 28:H804 (1989), herein incorporated by reference). Sustained contractions, to 2 gm tension, were induced with 1 μM epinephrine, after which the effects of t-PA and S-NO-tPA were tested. In certain experiments the guanylate cyclase inhibitor, methylene blue, was preincubated with vessel rings for 15 minutes prior to initiation of contractions.

3. Vascular Relaxations

As shown in the illustrative tracings of Figure 9, S-NO-t-PA, at pharmacologic concentrations, induces relaxations that are unmatched by equimolar amounts of the reactant protein-thiol or NO alone. Furthermore, consistent with the mechanism of other nitro(so)-vasodilators, relaxations were attenuated by the guanylate cyclase inhibitor, methylene blue. Table 3 depicts the effect of S-NO-t-PA on vessel relaxation for several such experiments.

Table 2

Kinetic Parameters of S2288 Hydrolysis and GLU-Plasminogen (S2251) Activation By t-PA and S-NO-t-PA

	$K_m/\mu M$	K_{cat} (sec^{-1})	K_{cat}/K_m ($sec^{-1}-\mu M^*$
S2288			
t-PA	280	0.52	0.0019
S-NO-t-PA	295	0.52	0.0019
S2251			
t-PA	3.5	0.200	0.056
S-NO-t-PA	3.8	0.262	0.069

Table 3

Vessel Relaxation

		% Relaxation
t-PA	(150 nM)	2.5 ± 4
NO	(150 nM)	1.0 ± 1.7
S-NO-t-PA	(150 nM)	20 ± 7*

Mean results (± S.D.; n=4) of vessel relaxation induced by S-NO-t-PA, and the comparable relaxation induced by equivalent concentrations of NO (generated from acidified $NaNO_2$) a t-PA.

* Relaxations to S-NO-t-PA were significantly greater than those induced by $NaNO_2$ or t-PA, as shown in this table for equal concentrations.

Table 4

Platelet Inhibition

% Normalized Extent Aggregation

		ADP (5μM)	Thrombin (0.024 U/ml)
t-PA	(150 μM)	1.06 ± 0.24	0.90 ± 0.15
S-NO-t-PA	(150 μM)	0.77 ± 0.28*	0.73 ± 0.28*

Mean results (± S.D.; n = 13-17) of platelet inhibition mediated by S-NO-t-PA to both AD-induced platelet aggregation. NO generated from $NaNO_2$ (150 nM) had no significant effect on platelet inhibition in these experiments (0.98 ± 0.11, n = 5).

p < 0.025 compared widi t-PA; p < 0.01 compared with t-PA.

Statistics

Determination of statistical significance was analyzed using a nonpaired t-test or two-way analysis of variance (ANOVA) followed by a Newman-Keul's comparison.

Example 7

Platelet Inhibitory and Vasodilatory Effect of S-Nitroso-BSA

A. Platelet Inhibition

The effect of S-nitroso-BSA on platelet aggregation was studied, using a gel-filtered platelet preparation, as previously described (Hawiger *et al., Nature, 2831:195* (1980)) and suspended at 150,000 platelets/μl in HEPES buffer, pH 7.35. S-NO-BSA was incubated with platelets for ten minutes at 37°C in a PAP-4 aggregometer (BioData, Hatboro, PA), after which aggregations were induced with 5 μM ADP. Aggregations were quantified by measuring the extent of change of light transmittance and expressed as a normalized value relative to control aggregations.

In control experiments, neither $NaNO_2$ at concentrations up to 15 μM nor the effluent from cells stimulated to secrete EDRF in the absence of BSA had any significant effect on either vessel tone or platelet aggregation. All non-nitrosylated proteins studied had no significant effect on platelet aggregation at any concentration tested.

Dose-dependent inhibition of ADP-induced platelet aggregation was observed over the range of 150 nM to 15 μM S-nitroso-protein. A nitrosylated protein plasma fraction was even more potent, manifesting inhibition at estimated -S-NO concentrations of 150 pM. S-nitroso-proteins synthesized with acidified $NaNO_2$, with NO gas, or by exposure to bovine aortic endothelial cells stimulated to secrete EDRF were essentially equipotent, as shown for S-nitroso-BSA in Figure 10. Furthermore, the platelet inhibitory effect of S-nitroso-BSA (1.4 μM) was confirmed both in platelet-rich plasma and in whole blood (using impedance aggregometry in this latter case) (Chong *et al., Drug Met. and Disp., 18:61* (1990) herein incorporated by reference).

Representative mean data and illustrative aggregation tracings for S-nitroso-BSA are provided in Figures 10 and 11a, respectively. Carboxyamidation of protein thiols with iodoacetamide or pretreatment of platelets with the guanylate cyclase inhibitor methylene blue abolished the antiplatelet effects of S-nitroso-proteins Figure 11a). In addition, the half-life of the antiplatelet effects correlated with that for vascular smooth muscle relaxation.

B. Vasodilation

1. Methods

The vasodilatory actions of S-nitroso-BSA were examined in a standard bioassay containing endothelium-denuded rabbit

aortic strips in Kreb's buffer, pH 7.5, at 37°, as described in Example 6.

2. Results

Dose-dependent relaxations were observed over the range of 15 nM to 15 μM S-nitroso-proteins, and representative mean data for S-nitroso-BSA are provided in Figure 10. S-nitroso-proteins synthesized with acidified $NaNO_2$, with NO gas, or by exposure to bovine aortic endothelial cells stimulated to secrete EDRF were essentially equipotent; this is again exemplified for S-nitroso-BSA in Figure 10. The relaxation response to S-nitroso-BSA proteins differed from that generally ascribed to EDRF, authentic NO, and the relatively labile low molecular weight biological S-nitrosothiols, all of which are characterized by rapid, transient relaxations. In marked contrast, S-nitroso-BSA induced a less rapid, but much more persistent, relaxation response (Figure 11b), thus confirming that it acts as a long-acting vasodilator.

Furthermore, BSA incubated with NO synthase in the presence of cofactors required for enzyme activity (calmodulin, NADPH, Ca^{++}) showed an L-arginine-dependent ability to induce persistent vasorelaxation characteristic of S-nitroso-proteins.

The half-life of S-nitroso-BSA as determined in the bioassay corresponded with chemical measurements of half-life and is approximately twenty-four hours. This half-life is significantly longer than the half-lives of low molecular weight S-nitrosothiols and suggests that the temporal profile of the relaxation response for S-nitrosothiols correlates with the liability of the S-NO bond.

Blockade of protein thiols by carboxyamidation with iodoacetamide prevented S-nitrosothiol formation as determined chemically, and rendered the proteins exposed to NO or EDRF biologically inactive (Figure 11b). Consonant with the mechanism of other nitro(so)-vasodilators (Ignarro, L.J. *Circ. Res.* 65:1 (1989)), relaxations were abolished by methylene blue, an inhibitor of guanylate cyclase (Figure 11a). This mechanism was confirmed by showing that S-nitroso-BSA (18 μM) induces 3.5-fold increases (n = 2) in cyclic GMP over basal levels relative to BSA alone in cultured RFL-6 lung fibroblasts containing a soluble guanylate cyclase exquisitely sensitive to NO (Forstermann *et al., Mol. Pharmacol.*, 38:7 (1990)). Stimulation of guanylate cyclase by S-nitroso-BSA was attenuated by methylene blue.

Figure 10 demonstrates the dose-dependent relaxation of vascular smooth muscle and inhibition of platelet aggregation with S-nitroso-BSA (S-NO-BSA). Dose-effect curves for vessel relaxation (▪–▪) and platelet inhibition (●–●) were generated with S-NO-BSA synthesized with equimolar NO generated from acidified $NaNO_2$ as described in the text and then neutralized to pH 7.4. Data are presented as mean ± SEM (n = 6-18). The open symbols represent experiments, in the vessel (□) and platelet (○) bioassays, in which S-NO-BSA was synthesized by exposure of BSA to bovine aortic endothelial cells stimulated to secrete EDRF. These data are presented as mean ± SEM (n = 3-8), with the X-axis error bars indicating the variance in the concentration of S-NO-BSA generated from EDRF and the Y-axis error bars indicating the variance in the bioassay response.

In vessel experiments, relaxations to S-NO-BSA are expressed as percent of tone induced by 1.0 μM norepinephrine.

Infusion of S-NO-BSA into anesthetized dogs, according to standard methods known in the art, resulted in prolonged decreases in blood pressure, unmatched by low molecular weight S-nitrosothiols. In addition, this compound increased coronary flow, thus preserving myocardial blood flow. In a canine model of subtotal coronary artery occlusion, S-NO-BSA inhibited platelet-dependent cyclic thrombus formation and significantly prolonged bleeding times. These extremely potent, but reversible anti-platelet properties *in vivo* are unmatched by classic nitrates. As well, the improvement in coronary blood flow contrasts markedly with the clinically used nitroso-compound, nitroprusside, which has deleterious effects on coronary flow. As shown in Figures 12-14, the constellation of anti-platelet effect, long duration of action, and increased coronary blood flow, is unmatched by other nitrosocompounds. Thus, S-nitroso-proteins have very unique hemodynamic and bioactive profiles.

Example 8

Vasodilatory Effect of S-Nitroso-Cathepsin

The effect of S-NO-cathepsin was studied according to the methods described in Example 7a. Results obtained demonstrated that S-NO-cathepsin, at a concentration of 150nM-1.5μM, inhibits platelet aggregation.

The effect of S-NO-cathepsin on vasodilation was studied according to the methods described in Example 7b. As shown in the illustrative tracings of Figure 12, S-NO-cathepsin, at a concentration of 150 nM - 1.5 μM induces vessel relaxation which is ummatched by equimolar amounts of non-nitrosylated cathepsin.

Example 9

Platelet Inhibitory and Vasodilatory Effect of S-Nitroso-Lipoprotein

The effect of S-NO-LDL on platelet aggregation was studied according to the methods described in Example 7a. Aggregations were quantified by measuring the extent of change of light transmittance, and expressed as a normalized value relative to control aggregations. As shown the illustrative tracings of Figure 13, inhibition of platelet aggregation is demonstrable at a concentration of 1 μM S-NO-LDL.

The effect of S-NO-LDL on vasodilation was studied according to the methods described in Example 7b. As shown in Figure 14, S-NO-LDL induces vessel relaxation which is unmatched by equimolar amounts of non-nitrosylated LDL.

Example 10

Platelet Inhibitory and Vasodilatory Effect of S-Nitroso-Immunoglobulin

The effect of S-NO-Ig on platelet aggregation was studied according to the methods described in Example 7a. Aggregations were quantified by measuring the extent of change of light transmittance, and expressed as a normalized value relative to control aggregations. As shown in Figure 15, inhibition of platelet aggregation is demonstrable at concentrations of S-NO-Ig in the pharmacologic range of 150 nM - 1.5 μM.

The effect of S-NO-Ig on vasodilation was studied according to the methods described in Example 7b. As shown in Figure 15, S-NO-Ig, at concentrations in the range of 150 nM - 1.5 μM, induces relaxation which is unmatched by equimolar amounts of immunoglobulin alone.

Example 11

Relaxation of Airway Smooth Muscle By S-Nitroso-BSA

1. Materials

Glutathione, L-cysteine, DL-homocysteine, D-penicillin, hemoglobin (bovine), methylene blue and Medium 199 sets were purchased from Sigma Chemical Co., St. Louis, MO. N-acetylcysteine was obtained from Aldrich Chemical Co., Milwaukee, WI. Captopril was kindly provided by Dr. Victor Dzau. Sodium nitrite, histamine and methacholine were purchased from Fisher Scientific, Fairlawn, N.J. Leukotriene D. was purchased from Anaquest, BOC Inc., Madison, WI. Antibiotic/antimycotic mixture (10,000 U/ml penicillin G sodium, 10,000 mg/ml, streptomycin sulfate, 25 mg/ml amphotericin B) was purchased from Gibco Laboratories, Grand Island, NY. Radioimmunoassay kits for the determination of cyclic GMP were purchased from New England Nuclear, Boston, MA.

2. Preparation of Airways

Male Hartley guinea pigs (500-600g) were anesthetized by inhalation of enflurane to achieve a surgical plane of anesthesia. The trachea were excised and placed in Kreb's-Henseleit buffer (mM); NaCl 118, KCl 5.4, NaH_2PO_4 1.01, glucose 11.1, $NaHCO_3$ 25.0, $MgSO_4$ 0.69, CaCl 2.32, pH 7.4. The airways were then dissected free from surrounding fat and connective tissue and cut into rings 2-4 mm in diameter. The trachea rings were, placed in sterile Medium 199 containing 1% antibiotic/antimycotic mixture in an atmosphere of 5% CO_2, 45% O_2, 55% N_2 and kept for up to 48 hours in tissue culture. The experiments were also performed on human airways isolated by the same method.

3. Bioassay

Trachea rings were mounted on stirrups and connected to transducers (Model FTO3C Grass), by which changes in

isometric tension were measured. Rings were then suspended in 10 cc of oxygenated (95% O_2, 5 % CO_2) buffer. Airway rings were equilibrated for 60 minutes under a load of 1 gm and then primed twice by exposure to 100 μM methacholine. The rings were contracted with various agonists at concentrations determined to generate 50% (± 16% S.D.) of maximum tone, after which the effect of S-NO-BSA was assessed. In selected experiments, relaxation responses were determined in the presence of hemoglobin, or after rings had been preexposed to methylene blue for 30 minutes.

4. Results

As shown in Figure 17, S-NO-BSA is a potent airway smooth muscle relaxant, producing 50% relaxation at a concentration of 0.01 μM and over 75% reaxation at a concentration of 10 μM.

Example 12
Inhibition of Enzymatic Activity of Cathepsin B by Nitrosylation

The enzymatic activity of S-NO-cathepsin B was measured against the chromogenic substrate, S2251 at pH 5, in sodium acetate buffer. S-nitrosylation resulted in a loss of enzymatic activity.

Example 13
Nitrosylation of Aromatic Amino Acids

1. Methods

a. Preparation of Nitroso-tyrosine

50 mmol of L-tyrosine (Sigma Chemical Company; St. Louis, MO) were dissolved into 0.5 ml of distilled water. 250 mmol of $Na^{15}NO_2$ (sodium N-[15]nitrite: MSD Isotopes, Merck Scientific; Rahway, NJ) were dissolved into 0.5 mL of 1 N HCl (Fisher Scientific; Fair Lawn, NJ) and transferred immediately to the aqueous tyrosine solution with agitation

by Vortex stirrer. The solution was capped and allowed to sit at room temperature for 30 minutes.

NMR measurements were made as follows:

(a) ^{15}N-NMR: D_2O was added and measurements were taken immediately;

(b) ^{1}H-NMR: After ^{15}N-NMR was completed, the solution was removed and placed into a small round-bottom flask. Water was removed *in vacuo*. D_2O was added to the dry off-white solid (this time as a solvent) and measurements were run immediately;

(c) Infrared Spectroscopy: Fourier Transform Infrared Spectroscopy (FTIR) samples were prepared through removal of water (as in (b)) and subsequent creation of a Nujol Mull using mineral oil.

(d) Ultraviolet and Visible Spectroscopy (UV-Vis): Samples for UV-Vis examination were used as per the above preparation without further modification. Samples were referenced to distilled water.

b. Nitrosylation of Phenylalanine, Tyrosine, and L-Boc-Tyr (Et)-OH.

50 mmol of L-phenylalanine, L-tyrosine (Sigma Chemical Company; St. Louis, MO), or L-boc-tyr(Et)-OH (Bachem Bioscientific Incorporated; Philadelphia, PA) were dissolved into 0.5 ml of distilled water. 250 mmol of $NA^{15}NO_2$ (sodium N-[15] nitrite) were dissolved into 0.5 ml of 1 N HCl (aq.) and transferred immediately to the aqueous amino acid solution with agitation by Vortex stirrer. The solution was capped and allowed to sit at room temperature for 30 minutes. ^{15}N-NMR and ^{1}H-NMR were performed as per nitroso-tyrosine above. Standard reference of tyrosine for FTIR was prepared as a Nujol Mull of pure crystalline L-tyrosine.

c. Nitrosylation of Tryptophan

1.7 mM of tryptophan were reacted with equimolar $NaNO_2$ in 0.5 N HCl for time periods of 5, 10, 15 and 60 minutes at 25°C.

2. Results

a. ^{15}N-NMR data

All NMR [^{15}N and ^{1}H] were run on two Bruker AM-500 MgHz spectrometers. Nitrosylation of tyrosine at pH 0.3 gives signals at approximately 730 ppm and 630 ppm relative to saturated sodium N-[15] nitrite aqueous solution referenced at 587 ppm ($^{15}NO_2$) (Fig. 21a.). A signal at 353 ppm (aqueous NO^{12}) was also observed. Nitrosylation of phenylalanine under the same conditions gave the signal at approximately 630 ppm but not the 730 ppm signal despite repeated attempts (Figure 22). Nitrosylation of phenylalanine also yielded signals at 587 ppm (excess, unprotonated nitrite) and 353 ppm. Nitrosylation of 0-blocked tyrosine model, boc-tyr(Et)-OH, also yielded a signal at approximately 630 ppm; and others at 587 ppm and 353 ppm. Small signals in the range 450-495 ppm were observed for the tyrosine models, phe and boc-tyr(E+)-OH.

b. ^{1}H-NMR data

To further characterize the nitrosylation of the phenolic functionality of L-tyr to the exclusion of C-nitrosylation, proton-NMR was performed on nitrosylatetyrasine; modification of L-tyr at the phenolic-OH would not appear in proton-NMR because of proton exchange with the deuterated solvent (D_2O). Examination of the spectra showed the classic doublet of doublets at low field, which is characteristic of para-disubstituted benzene, thus excluding aromatic proton substitution (Figure 21b). This, and other values in the spectra were characteristic of unmodified L-tyr.

c. FTIR data

All FTIR were run on a Nicolet 5ZDX FT-IR Spectrometer. FTIR of a Nujol Mull of L-tyrosine showed a very characteristic and well-documented alcoholic stretch in the spectra due to the phenolic-OH (Figure 1d. inlaid). This spectrum lacked any signal(s) at the 1680-1610 cm^{-1} range that coincides with the O-N=O stretch (not shown). FTIR of nitrosylated L-tyrosine showed no evidence of alcoholic-OH stretches and contained two small bands in the range of 1680-1610 cm^{-1} that could possibly account for the expected O-N=O stretch (Wade, L.G., *Organic Chemistry (1st Ed.)* Prentice-Hall Inc., Englewood Cliffs, N.J.: 1987. p. 1334) (Figure, 21c.).

d. UV-Vis data

All UV-Vis spectroscopy was performed using a Gilford Response UV-Vis Spectrophotometer (CIBA-Corning, Oberlin, OH). Treatment of L-tyrosine with aqueous sodium nitrite at pH 0.3 (0.5N HCl) resulted in a yellow solution with an absorption maximum at 361 nm. This result is similar to, but differs from previously reported results with nitrosated L-tyrosine. Ortho-ring substituted L-nitro-tyrosine (Sigma) absorbs at 356 nm at pH 0.3.

Treatment of phenylalanine with sodium nitrite at pH 0.3 gives a rapidly changing UV spectrum with a peak increasing in wavelength from 318 run at 5 min. to a maximum unchanging peak at 527 nm by 30 min.

Figure 23(a-e) demonstrates time-dependent nitrosylation of tryptophan. The data is suggestive of nitrosylation of both the aromatic ring and amino groups.

Example 14

Nitrosylation of BSA

BSA, at 200 mg/ml, was loaded at a ratio of 20:1 with NO in 0.5 N HCl for 30 minutes at room temperature. As shown in Figure 24, the 726 ppm peak indicates O-nitrosation of the tyrosine residues on BSA. Figure 24 also provides evidence for the nitrosation of several other functional groups on BSA. The data are also suggestive of ring nitrosation and amine nitrosation (600 ppm peak) as well.

Time-dependent NO loading of BSA was performed by exposing BSA (200 mg/ml) in phosphate buffer (10 mM, pH 7.4) to NO gas bubbled into the BSA solution, for 1,5 and 30 minute time periods. Figure 25 provides UV spectrum data which indicates NO loading of BSA.

Example 15

Nitrosylation of t-PA: NO Loading

t-PA at 10 mg/ml was exposed 10:1 to excess $NaNO_2$ in 0.5 N HCl. Figure 26 shows NO-loading of t-PA.

Example 16

Vasodilatory Effect of NO-Loaded BSA

BSA was loaded with NO according to the method described in Example 14. Vasodilatory effect was studied in a rabbit aorta bioassay, according to the methods described in Example 6C. As shown in Figure 27, increasing concentrations of NO resulted in an increase in vessel relaxation induced by the resultant NO-BSA.

Example 17

Guanylate Cyclase Inhibitors Do Not Inhibit S-nitroso-protein-Induced Relaxation in Human Airways

The effect of guanylate cyclase inhibitors upon S-nitroso-protein-induced airway relaxation and cGMP increase

was assessed, using the previously described bioassay and cyclic nucleotide assay procedures. The bronchodilatory effect of S-nitroso-albumin was examined in human airways (5-12 mm outer diameter). Concentration-response relationships for rings contracted with methacholine (7 μM) resulted in IC50 values of 22 μM, approximately two orders of magnitude greater than theophylline.

S-nitroso-albumin (100 μM) induced increases over control airway cGMP levels. However, S-nitroso-albumin-induced airway relaxation was not significantly inhibited by methylene blue (10^{-4}) or LY83583 (5 x 10^{-5}). Similarly, hemoglobin (100 μM) had little effect on S-nitroso-albumin-induced relaxation (P = NS).

These results demonstrate that the mechanism by which S-nitroso-protein cause airway relaxation is not due solely to increases in cGMP. Thus, S-nitroso-proteins cause airway relaxation through both an increase in cyclic GMP, as well as a cGMP-independent pathway. In doing so, they provide a means for achieving combination therapy by maximizing the synergistic effect of two separate mechanisms.

Example 18

S-nitroso-Proteins Resist Decomposition in the Presence of Redox Metals

The stability of S-nitroso-albumin in the presence of oxygen and redox metals was assessed. When subjected to conditions consisting of 95% O_2, pH 7.4, the half life of this compound was shown to be on the order of hours, and significantly greater than that of NO, or NO_2, which, under similar conditions, are on the order of seconds.

In addition, S-nitroso-protein stability was assessed in the presence of various redox metals or chelating agents. S-nitroso-albumin was resistant to decomposition when Cu^{+}, Fe^{2}, or Cu_{2+} (50 μM) or defuroxamine or EDTA (10 μM) were added. Thus, these experiments demonstrate that, unlike NO•, S-nitroso-proteins are not rapidly inactivated in the presence of oxygen, nor do they decompose in the presence of redox metals.

Example 19
S-nitrosylation of Hemoglobin Increases Hemoglobin-oxygen Binding

Additional experiments were conducted to evaluate the reaction between S-nitrosothiols and hemoglobin. S-nitrosylation of hemoglobin was accomplished by reacting 12.5 μM hemoglobin with 12.5 μM acidified $NaNO_2$ or an RS-NO compound such as S-nitroso-N-acetylcysteine, S-nitroso-glutathione or S-nitroso-cysteine for 5 and 20 minute intervals (pH 6.9). S-nitrosylation was verified, using standard methods for detection of S-nitrosothiols (Saville, *Analyst*, 83:670-672 (1958)). The Saville method, which assays free NO_x in solution, involves a diazotization reaction with sulfanilamide and subsequent coupling with the chromophore N-(1-naphthyl)ethylenediamine. The specificity for S-nitrosothiols derives from assay determinations performed in the presence and absence of $HgCl_2$, the latter reagent catalyzing the hydrolysis of the S-NO bond. Confirmatory evidence for S-nitrosothiol bond formation was obtained by spectrophotometry, demonstrated by the absorption maximum of 450 nm, as shown in Figure 28. This was demonstrated using NO^{+} equivalents in the form of SNOAC.

As demonstrated by Figure 29, the UV spectrum of hemoglobin incubated with SNOAC shows no reaction at the redox metal (iron-binding site) of hemoglobin, over 15

minutes. For purposes of comparison, equimolar concentrations of hemoglobin and $NaNO_2$ were reacted in 0.5 N HCl, to form nitrosyl-hemoglobin, and the UV spectrum was obtained. As shown in Figure 30, NO reacted instantaneously with the redox metal site on hemoglobin. The fact that the S-nitrosothiol did not react with the redox metal site of hemoglobin, but with its thiol group instead, indicates that the reactive NO species donated by the S-nitrosothiol is nitrosonium or nitroxyl.

S-nitrosylation of hemoglobin does not result in the formation of methemoglobin and consequent impairment in hemoglobin-oxygen binding. Furthermore, an additional experiment demonstrated that S-nitrosylation of hemoglobin causes a leftward shift in the hemoglobin-oxygen association curve, indicating an increase in oxygen binding. Thus, the reaction between S-nitrosothiols and hemoglobin not only eliminates the inhibition of oxygen binding which occurs from the reaction with uncharged NO and generation of methemoglobin, but it actually increases oxygen binding.

Example 20
S-Nitrosylation of Hemoglobin

Our aim in these experiments was to design conditions which support selective S-nitrosylation of hemoglobin's β93 sulfhydryl without impairment of the oxygen delivery functionality. Our results indicate the feasibility of this reaction under physiological conditions (Figure 31). This is achieved by treating hemoglobin with agents which preferentially donate NO^+ (which targets SH groups), rather than NO● (which reacts preferentially with metals).

Hemoglobin is S-nitrosated by incubation with an alkylnitrite (e.g., amyl nitrite or tert-butyl nitrite) or a

thionitrite (e.g., S-nitroso-glutathione, S-nitroso-penicillamine, S-nitroso-cysteine and S-nitroso-N-acetylcysteine) for periods of one minute to one hour. The pH is optimized within the range of 6.5 to 7.5 to achieve stoichiometric S-nitrosylation. To avoid the potential formation of methemoglobin the reactions are preferably performed anaerobically. It may also be desired to perform the above procedure in saturating carbon monoxide solutions and under increased atmospheric pressures (i.e., 5 atmospheres) to prevent interaction of NO with the heme site.

Figure 31 shows the UV spectrum of S-NO-hemoglobin (Hb). The spectra show that oxygen binding to the heme site is largely unaffected at S-NO/Hb stoichiometries (0.05 and 0.37) that are lacking in smooth muscle contractile activity. Higher ratios of S-NO/Hb (1.59) are associated with methemoglobin formation. Curves for oxy-Fe(II)-hemoglobin (dithionite-treated) and methemoglobin ($K_3Fe(CN)$-treated) are shown for comparison.

Synthesis of S-NO-hemoglobin is monitored by three complementary methods, two of which were developed in this laboratory: photolysis-chemiluminescence and capillary electrophoresis methodologies. The ligand binding to heme is determined spectrophotometrically, using published extinction coefficients. The structure of S-nitrosylated hemoglobin is then further characterized using SDS, polyacrylamide gel electrophoresis and isoelectric focusing immunoelectrophoresis.

The photolysis-chemiluminescence technique (U.S. patent application serial number 07/872,237 filed April 22, 1992) can be used to measure both protein and low molecular weight RS-NO. The sample protein is introduced directly or as a chromatographic effluent from an attached FPLC/HPLC (for

separation and identification of protein and amino acid RS-NO, respectivelY) into a photolysis cell where it is irradiated with a 200 watt mercury vapor lamp, designed to result in complete photolysis of the S-N bond. NO is then carried in a stream of helium towards the reaction chamber which yields the chemiluminescence signal. To further ensure that signal originate from RS-NO, measurements are compared before and after treatment with $HgCl_2$, which selectively displaces NO from thiol groups. We have confirmed that metal-nitrosyl complexes are not affected by this treatment.

In the Saville assay method, NO^+ displaced from RSNO with Hg^{2+} ion is assayed by diazotization of sulfanilamide and subsequent coupling to the chromophore N-[1 naphthyl] ethylenediamine.

The capillary electrophoresis method (U.S. Patent No. 5,346,599) offer the added advantage of being able to separate hemoglobin variants as well as various RS-NO from their respective thiols and disulfides. We have recently reported on simultaneous detection of RS-NO derivatives of cysteine, glutathione and homocysteine, together with their respective thiols and disulfides, using this technique.

Example 21

Bioassays of S-NO-Hemoglobin

The adverse clinical consequences of cell-free hemoglobin results from its contractile effects on blood vessels, airways, and intestinal smooth muscle. *In vitro* bioassays are, therefore, used to assess the smooth muscle relaxant properties of S-NO hemoglobin in these tissues. Such organ chamber experiments are routinely performed to assess the effects of NO donors on blood vessels, airways and intestinal tissues.

Methods for preparation of blood vessels, airways and intestinal smooth muscle are performed as previously described. Briefly, descending thoracic aorta, trachea, or intestinal tissues are isolated from anesthetized New Zealand white female rabbits. The tissues are cleaned, cut into small rings and mounted on stirrups connected to force transducers by which changes in isometric tension are recorded. Sustained contractions are elicited with agonists (i.e., histamine or acetylcholine for airway smooth muscle or phenylephrine in the case of blood vessels), or by electrical field stimulation.

Preliminary experiments showing that S-NO-hemoglobin is a potent relaxant of phenylephrine contracted aortic rings is shown in Figure 32.

Figure 32 shows reversal of hemoglobin (Hb)-induced contraction of aortic rings by S-nitrosylation. Hemoglobin is shown to constrict vessels in a dose-dependent manner (●). S-NO-Hb, with a stoichiometry of 0.1 S-NO\Hb, entirely prevents the constrictor response (O). Increasing the S-NO-Hb\Hb stoichiometry to 1 converts hemoglobin into a potent vasodilator (+). Moreover, these studies show that S-nitrosylation of hemoglobin abrogates the contraction induced by the native protein.

Example 22

Oxygen Affinity of S-NO-Hemoglobin

The successful development of hemoglobin based blood substitutes involves an understanding of oxygen binding capability. In addition to classical influences on oxygen affinity (i.e., 2, 3, DPG, pH and Co_2), alkylation of the β93 SH group(s) of hemoglobin shifts the O_2 hemoglobin association curve leftward. For this, we evaluate the effects of S-nitrosylation on native and cross-linked hemoglobins, as well

as other hemoglobin variants. A comparative analysis of the effects of S-nitrosylation, Alkylation (i.e., with N-ethulmaleimide) or oxidation (i.e., with reactive disulfides such as dithionitrobenzoic acid) advance our understanding of the molecular control mechanisms of oxygen delivery.

Measurement of highly precise oxygen binding curves is performed using standard tonometry, an Imai cell and/or a thin-layer Gill cell modified with an oxygen electrode. For studies of oxygen binding by intact red blood cells, with or without added cell-free hemoglobins, a thin layer dual-beam method is used. For these measurements we use a modified Hemoscan (American Instruments Co.) operated in a discontinuous mode to avoid dynamic error.

Example 23

***In Vivo* Vasorelaxant Studies**

Rabbits are routinely used in the laboratory for assessment of hemodynamic responses to pharmacological agents. In addition, we use a superfused guinea pig lung model to assess the effects of NO donors on airway resistance. It is well known that hemoglobin infusions increase blood pressure in rabbits and would be predicted to modestly increase airway tone.

Our objectives were, therefore, to demonstrate vasorelaxant and bronchodilator properties of S-NO-hemoglobin, determine the biological half life of the molecule and obtain insight into the degree of S-nitrosation required to overcome its contractile effects. In particular, the data obtained in ring studies *in vitro* indicates that very small degrees of S-nitrosation are required to ameliorate vasoconstriction. Specifically, one NO per 40 heme groups is sufficient to prevent aortic smooth muscle contraction.

Hemodynamic Measurements

New Zealand white rabbits were anesthetized with ketamine (50 mg/kg i.m.) and sodium barbital (5-10 mg/kg i.v.) after which the femoral artery was cannulated to allow continuous measurement of blood pressure. Mean arterial pressure was then measured in response to intravenous bolus injections of S-NO hemoglobin (1 nmol/kg/min to 1 μm/kg/min) as well as continuous infusions.

Measurements of S-NO Hemoglobin in Blood

The kinetics of S-NO-hemoglobin decomposition *in vivo* was measured by photolysis-chemiluminescence, as previously described. Blood samples, anticoagulated with 0.13 M trisodium citrate, were obtained for analysis at 5 minutes intervals after injection. The red cells were lysed in deionized water, passed across a G25 column at 4°C to remove glutathione, and the S-NO-Hb content was then measured directly and is shown in Figure 33.

Figure 33 shows endogenous levels of S-NO-hemoglobin in rat artery, rat vein and human vein. Rat arterial blood was obtained by direct cardiac puncture, rat venous blood from the jugular vein and human blood by venipuncture, according to approved protocols.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is

intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A compound comprising a blood substitute to which is directly or indirectly linked an NO or NO_2 group.

2. The compound comprising a blood substitute of claim 1 wherein the blood substitute is a heme protein.

3. The compound comprising a blood substitute of claim 2 wherein the heme protein is a hemoglobin.

4. The compound comprising a blood substitute of claim 3 wherein the hemoglobin is human hemoglobin.

5. The compound comprising a blood substitute of claim 3 wherein the hemoglobin is bovine hemoglobin.

6. The compound comprising a blood substitute of claim 1 wherein the blood substitute is a modified hemoglobin.

7. The compound of claim 1 selected from the group consisting of an S-nitroso, N-nitroso, O-nitroso and C-nitroso blood substitute.

8. The compound of claim 7 wherein the blood substitute is a heme protein.

9. A blood substitute composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier therefor.

10. A blood substitute composition comprising the compound of claim 2 and a pharmaceutically acceptable carrier therefor.

11. A blood substitute composition comprising the compound of claim 3 and a pharmaceutically acceptable carrier therefor.

12. A blood substitute composition comprising the compound of claim 4 and a pharmaceutically acceptable carrier therefor.

13. A blood substitute composition comprising the compound of claim 5 and a pharmaceutically acceptable carrier therefor.

14. A blood substitute composition comprising the compound of claim 6 and a pharmaceutically acceptable carrier therefor.

15. A blood substitute composition comprising the compound of claim 7 and a pharmaceutically acceptable carrier therefor.

16. A blood substitute composition comprising the compound of claim 8 and a pharmaceutically acceptable carrier therefor.

17. The composition of claim 9 which further includes an additional component selected from the group consisting of a nonionic surfactant, a phospholipid, an emulsifier and a fatty acid.

18. The composition of claim 12 which further includes an additional component selected from the group consisting of a nonionic surfactant, a phospholipid, an emulsifier and a fatty acid.

19. A blood substitute composition comprising an emulsion of perfluorocarbon particles in a pharmaceutically acceptable carrier and which further comprises nitric oxide or a compound capable of donating, releasing or transferring nitric oxide.

20. The composition of claim 19 wherein the compound capable of donating, releasing or transferring nitric oxide is a nitrosothiol.

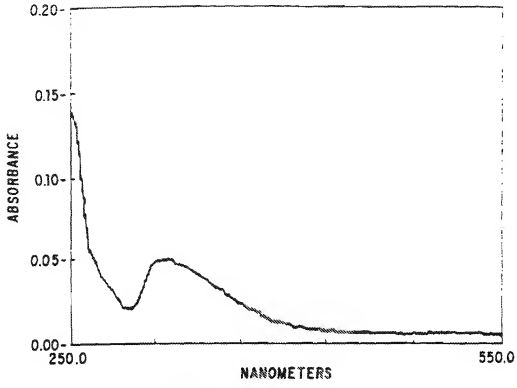

0.20
0.15
0.10
0.05
0.00
ABSORBANCE
250.0
550.0
NANOMETERS

FIG. 1A

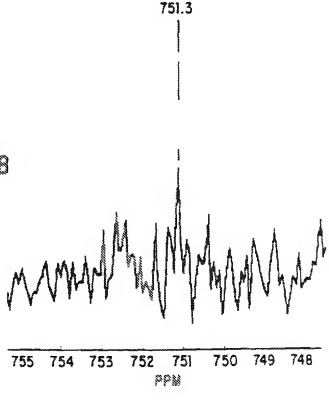

751.3
755 754 753 752 751 750 749 748
PPM

FIG. 1B

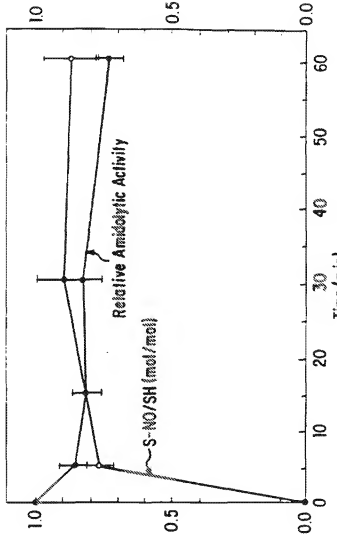

1.0
0.5
0.0
0
10
20
30
40
50
60
Time (min)
Relative Amidolytic Activity
S-NO/SH (mol/mol)

FIG. 2

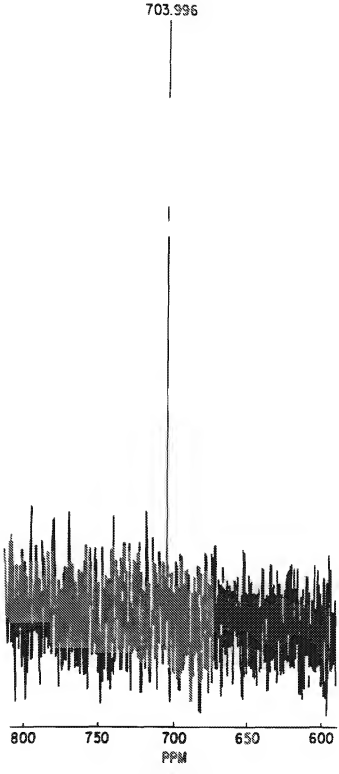


FIG. 3

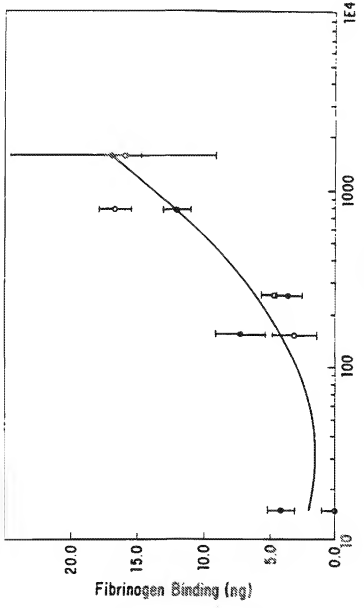

Fibrinogen Binding (ng)
20.0
15.0
10.0
5.0
0.0
10
100
1000
1E4
[Enzyme], nM

FIG. 4

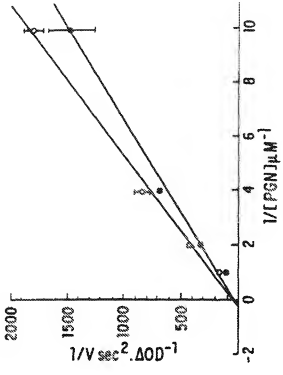

2000
1500
1000
500
1/V sec^2.ΔOD^{-1}
-2
0
2
4
6
8
10
1/[PGN]μM^{-1}

FIG. 5B

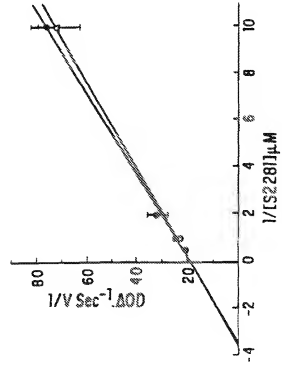

80
60
40
20
1/V Sec^{-1}.ΔOD
-4
-2
0
2
4
6
8
10
1/[S2281]μM

FIG. 5A

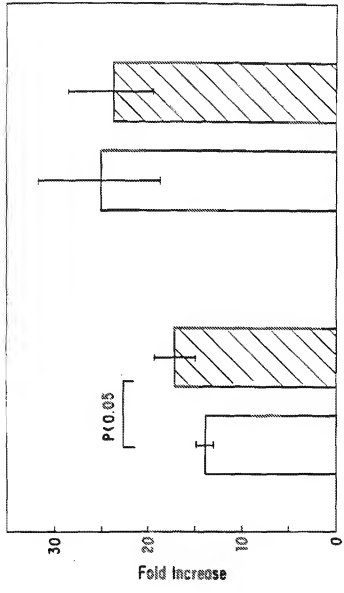


FIG. 6

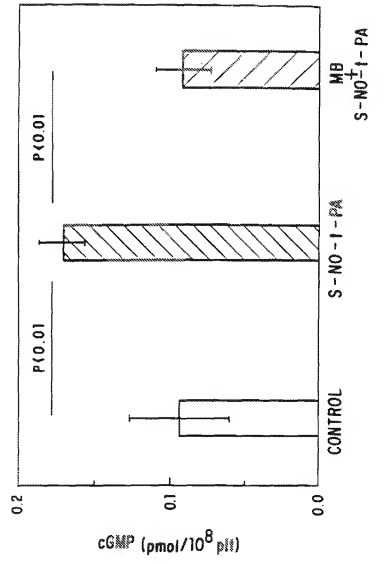


FIG. 7

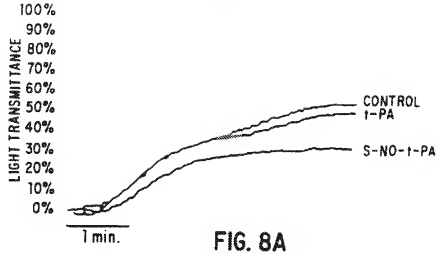


FIG. 8A

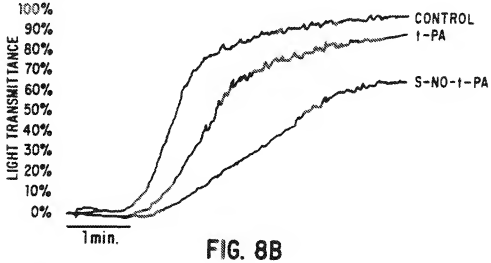


FIG. 8B

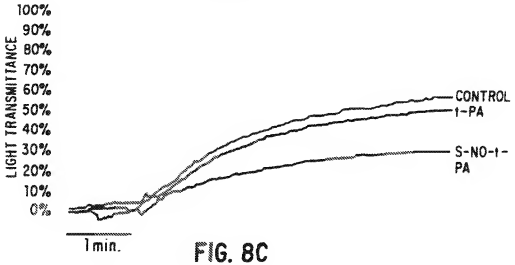


FIG. 8C

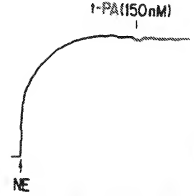


FIG. 9A

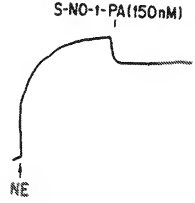


FIG. 9B

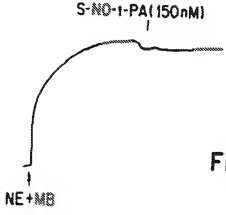


FIG. 9C

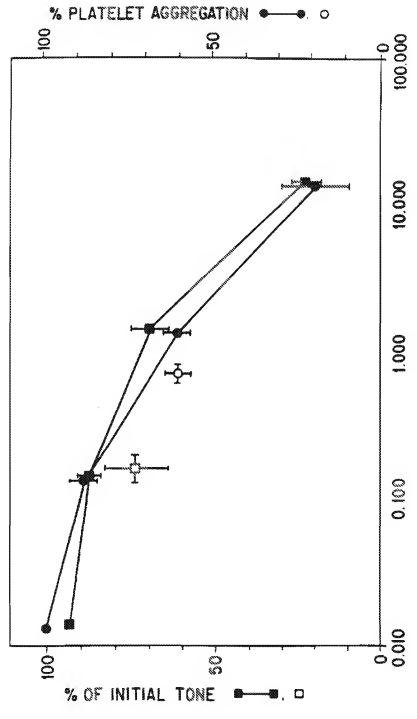

% PLATELET AGGREGATION
100
50
0
% OF INITIAL TONE
100
50
0
0.010
0.100
1.000
10.000
100.000
(S-NO-BSA),μM

FIG. 10

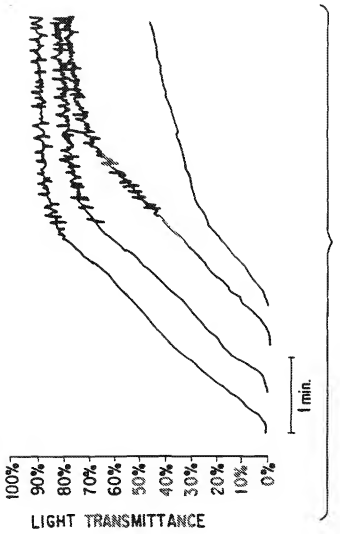


FIG. 11A

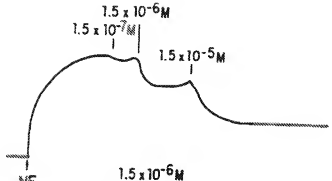


FIG. 11BA

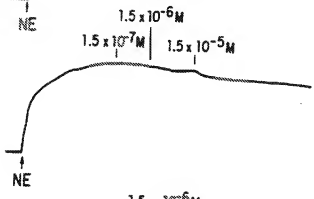


FIG. 11BB

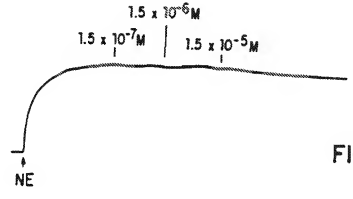


FIG. 11Bc

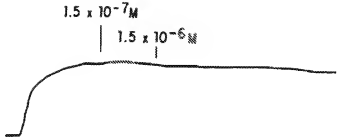


FIG. 11BD

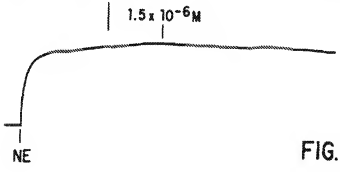


FIG. 11BE

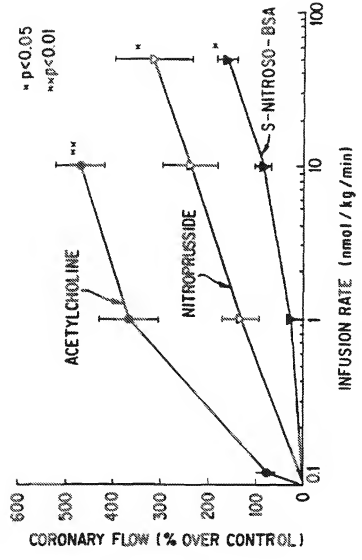

* p<0.05
** p<0.01
ACETYLCHOLINE
NITROPRUSSIDE
S-NITROSO-BSA
CORONARY FLOW (% OVER CONTROL)
INFUSION RATE (nmol/kg/min)
600
500
400
300
200
100
0
0.1
1
10
100

FIG. 12

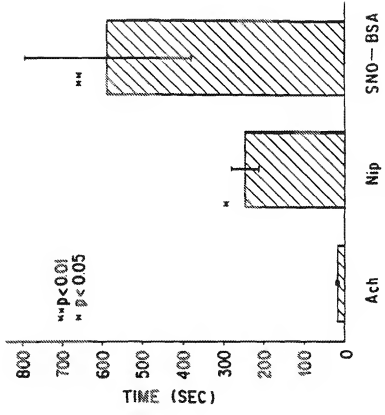

TIME (SEC)
800
700
600
500
400
300
200
100
0
**p<0.01
* p<0.005
Ach
Nip
SNO—BSA

FIG.13

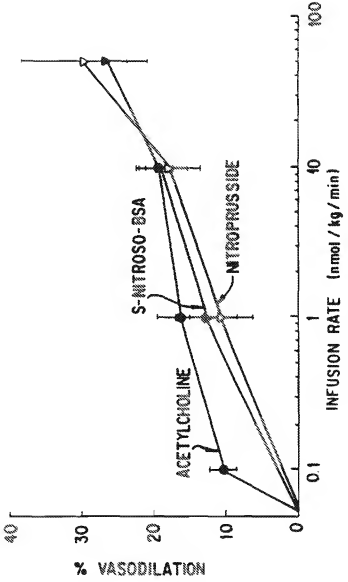

% VASODILATION
0
10
20
30
40
0.1
1
10
100
INFUSION RATE (nmol/kg/min)
ACETYLCHOLINE
S-NITROSO-BSA
NITROPRUSSIDE

FIG. 14

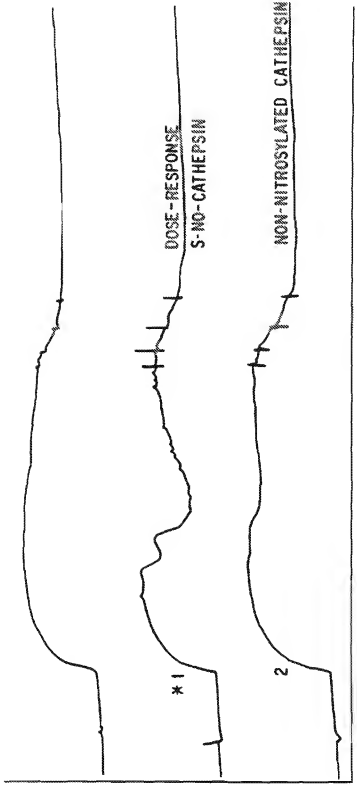

DOSE-RESPONSE
S-NO-CATHEPSIN
NON-NITROSYLATED CATHEPSIN
*1
2
*1 DOSE-RESPONSE TO S-NITROSO-CATHEPSIN

FIG. 15

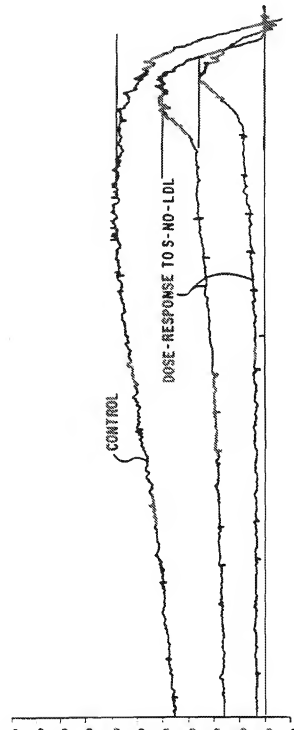

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
CONTROL
DOSE-RESPONSE TO S-NO-LDL
1 MINUTE
1 MINUTE
PLATELET INHIBITION

FIG. 16

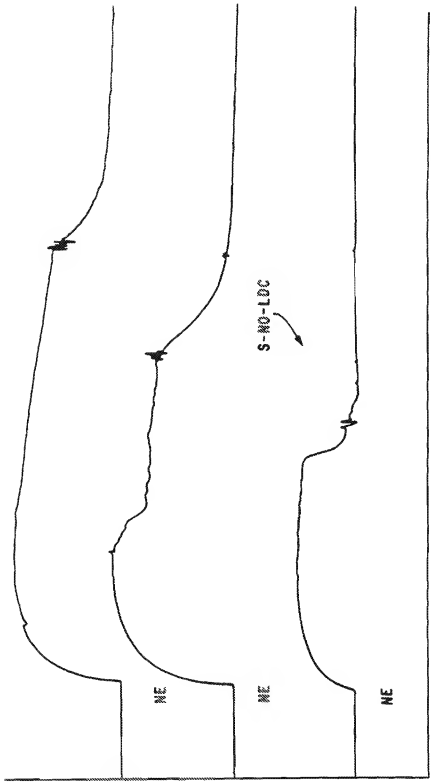


FIG. 17

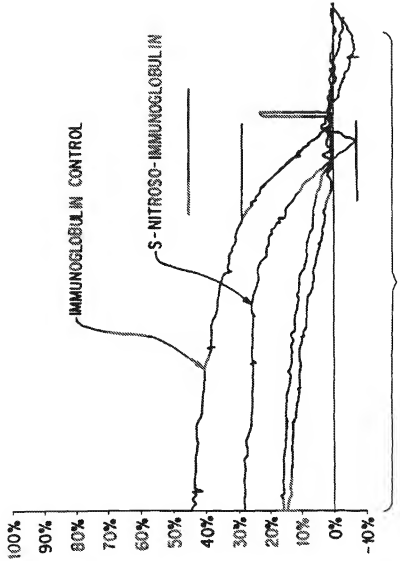

IMMUNOGLOBULIN CONTROL
S-NITROSO-IMMUNOGLOBULIN
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
1 MINUTE

FIG. 18

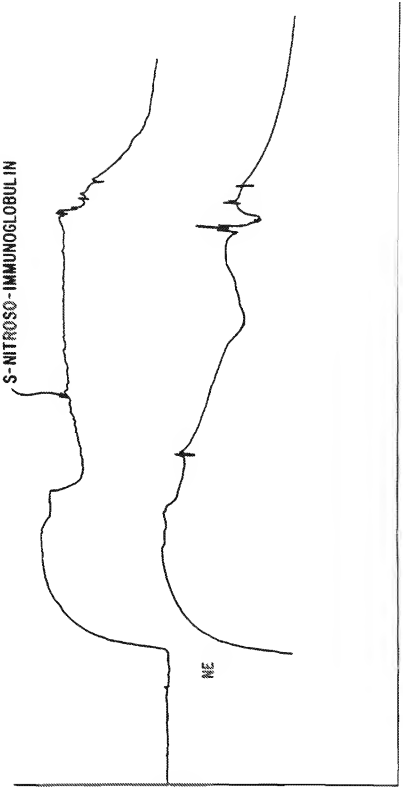


FIG. 19

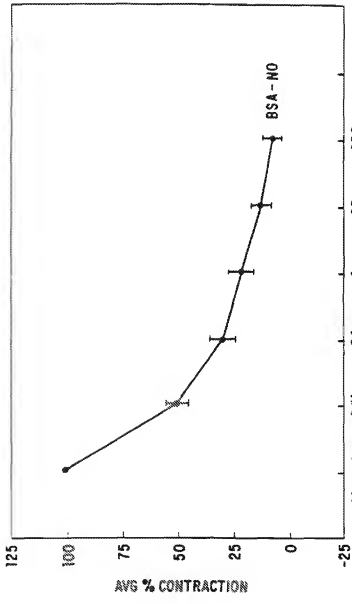

125
100
75
50
25
0
-25
AVG % CONTRACTION
BSA-NO
histamine contraction
0.01
0.1
1
10
100
CONCENTRATION IN MICROMOLAR

FIG. 20

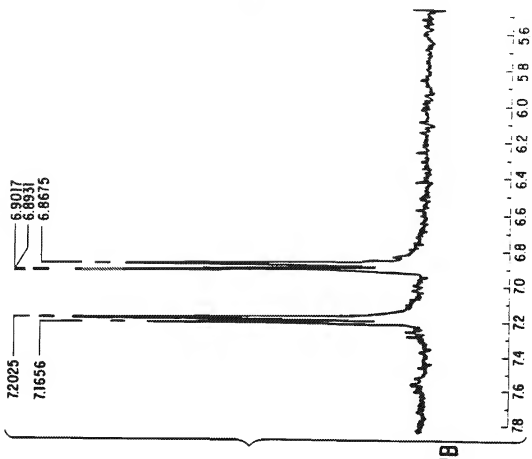


FIG. 21B

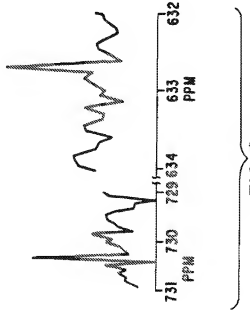


FIG. 21A

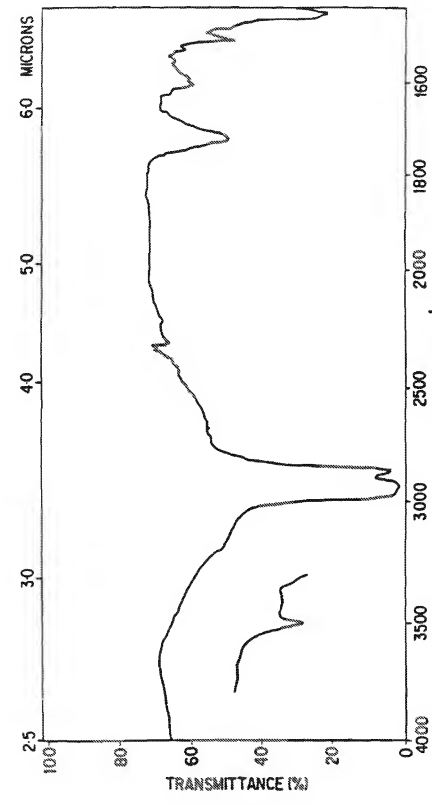

TRANSMITTANCE (%)
100
80
60
40
20
0
4000
3500
3000
2500
2000
1800
1600
WAVENUMBER (CM⁻¹)
2.5
3.0
4.0
5.0
6.0
MICRONS

FIG.21C

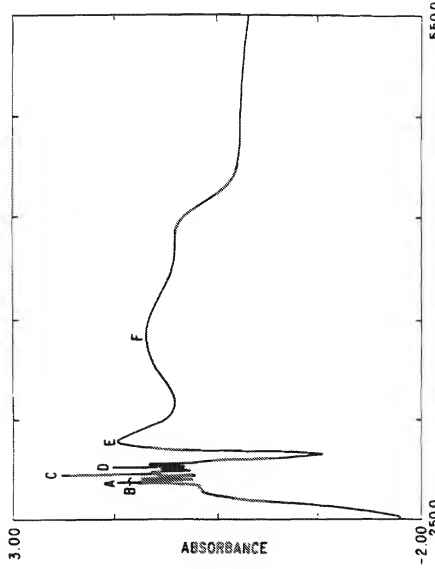


FIG. 21D

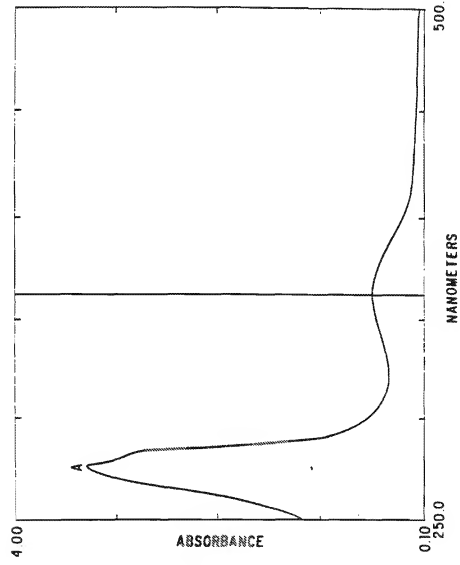


FIG. 21E

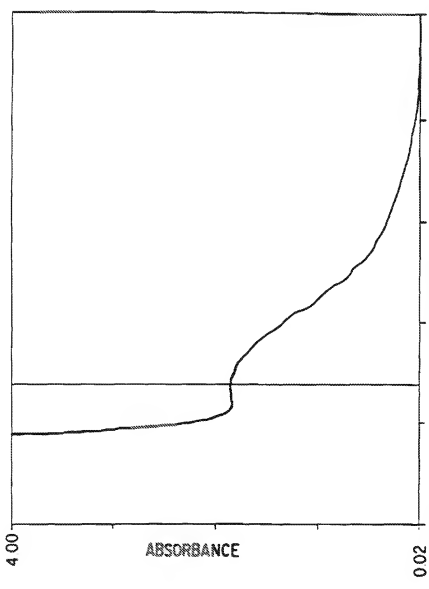


FIG. 23A

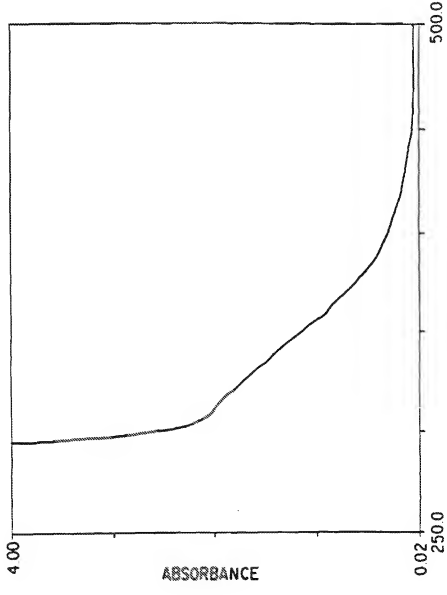


FIG. 23B

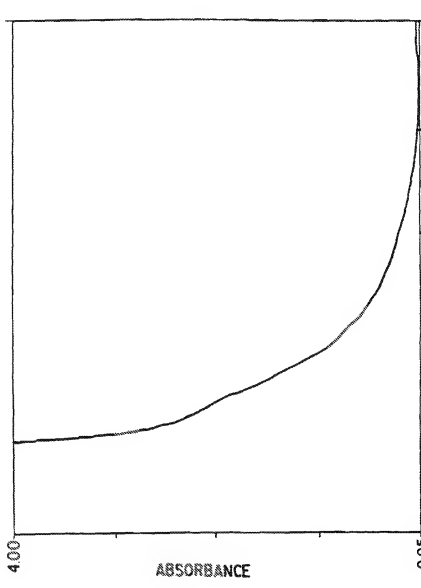


FIG. 23C

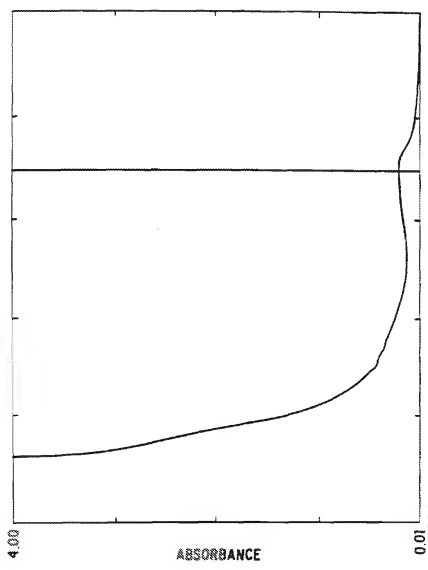


FIG. 23D

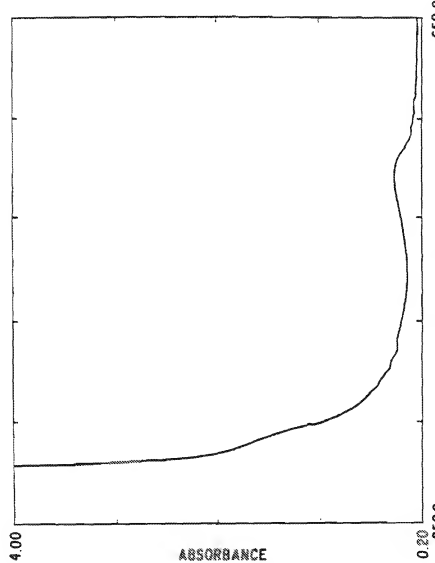


FIG. 23E

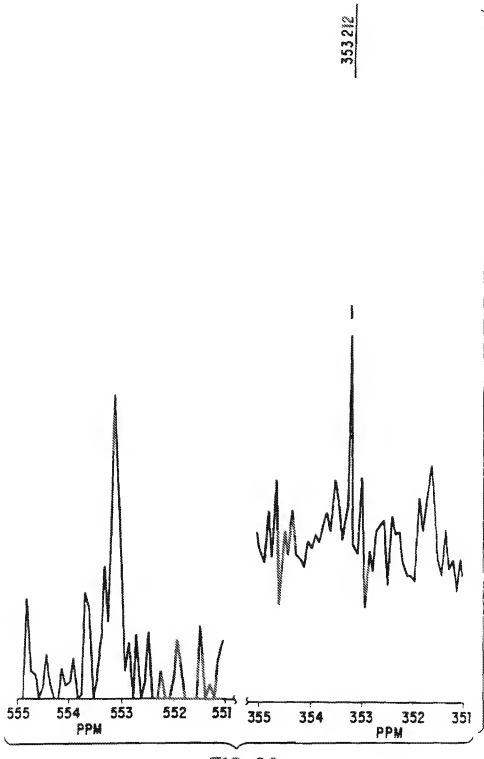


FIG. 22

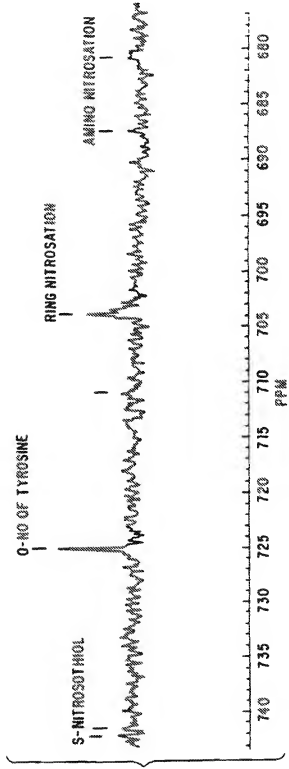

O-NO OF TYROSINE
RING NITROSATION
S-NITROSOTHIOL
AMINO NITROSATION
740 735 730 725 720 715 710 705 700 695 690 685 680
PPM

FIG. 24

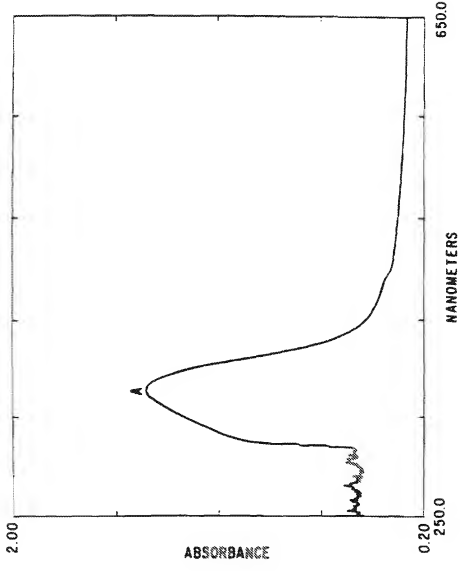


FIG. 25A

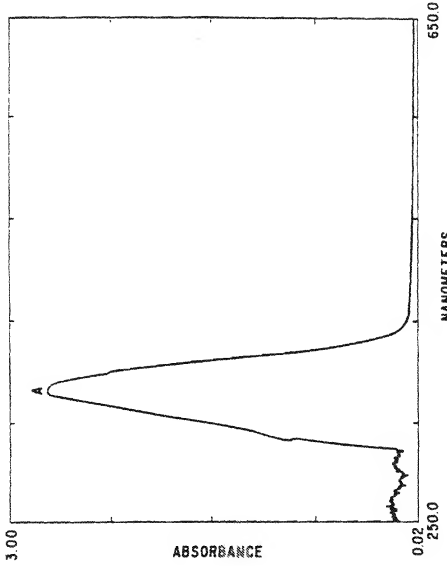


FIG. 25B

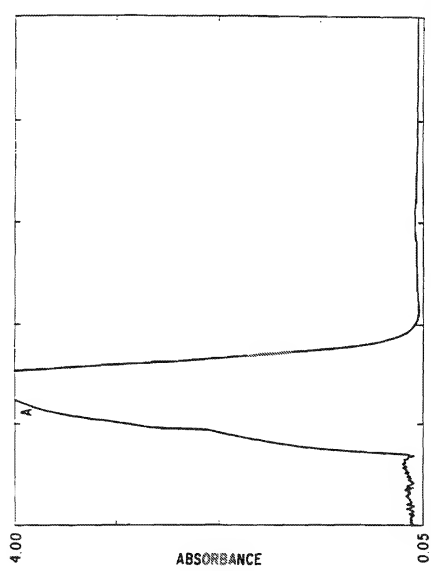


FIG. 25C

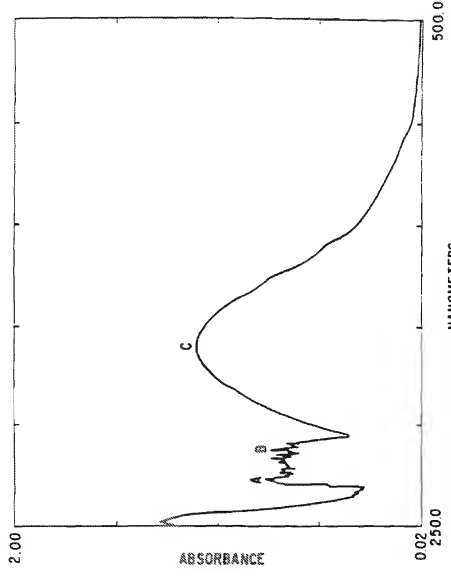
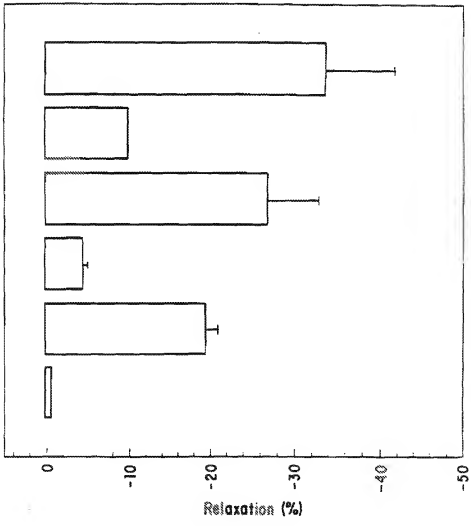


FIG. 26

FIG. 27


0
-10
-20
-30
-40
-50
Relaxation (%)
NO 153uM
BSA/NO 1:1
NO 1.5uM
BSA/NO 1:10
NO 15uM
BSA/NO 1:100

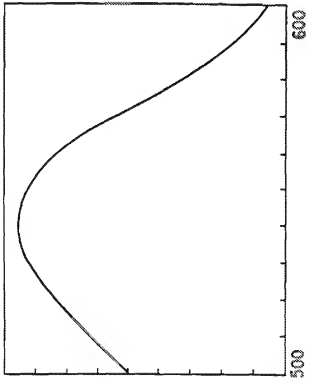


FIG. 28

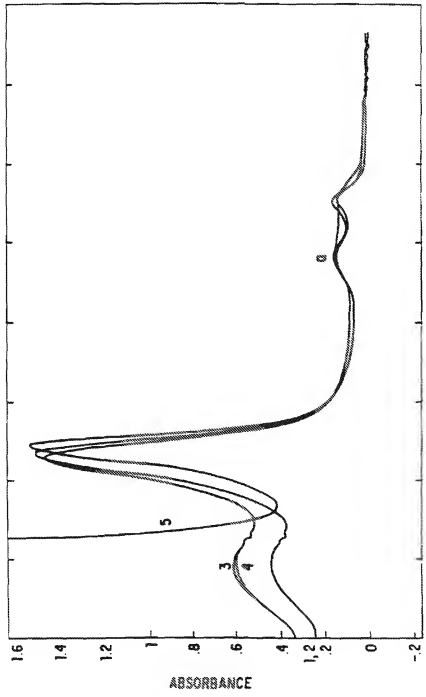

ABSORBANCE
1.6
1.4
1.2
1
.8
.6
.4
.2
0
-.2
300
350
400
450
500
550
600
650
WAVELENGTH (nm)
5
3
4
2

FIG. 29

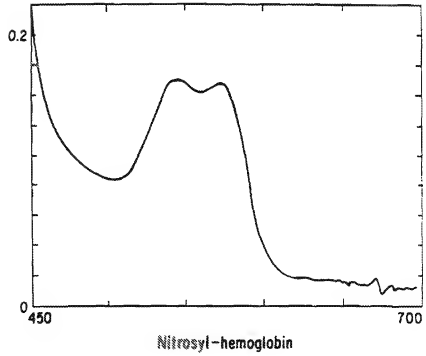


FIG. 30

FIG. 31

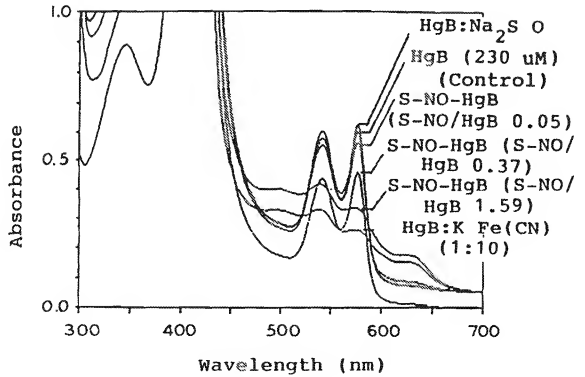

1.0
0.5
0.0
Absorbance
300
400
500
600
700
Wavelength (nm)
HgB:Na2S O
HgB (230 uM)
(Control)
S-NO-HgB
(S-NO/HgB 0.05)
S-NO-HgB (S-NO/
HgB 0.37)
S-NO-HgB (S-NO/
HgB 1.59)
HgB:K Fe(CN)
(1:10)

FIG. 32

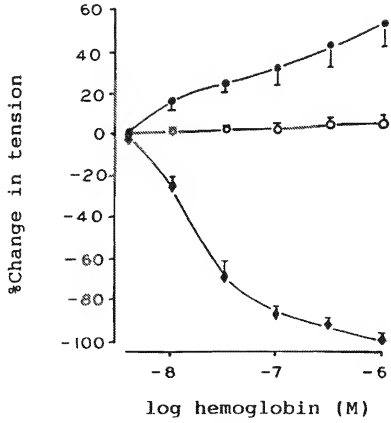
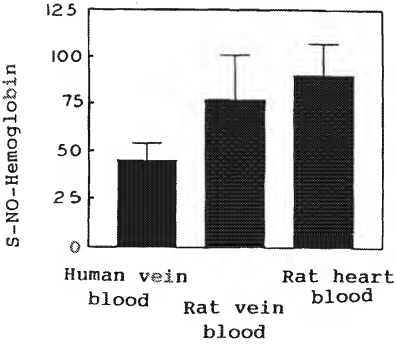

60
40
20
0
-20
-40
-60
-80
-100
%Change in tension
-8
-7
-6
log hemoglobin (M)

FIG. 33


125
100
75
50
25
0
S-NO-Hemoglobin
Human vein blood
Rat vein blood
Rat heart blood

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03866

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/14, 31/715, 31/ 765, 38/16; C07D 307/82
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/6, 231.2, 231.5, 231.8, 315, 316 , 451, 461, 747, 755, 759, 788, 832; 530/385

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ------ Y	Archives Biochem & Biophys, vol 303, No. 2, issued June 1993, "Nitric Oxide Binding to Human FerrihemoglobinsCross Linked between Either a or b Subunits." pages 332-338, see enitre disclosure teaching NO binding to hemeglobins.	1-18 -------- 19-20
X	US, A, 4,609,383 (BONAVENTURA ET AL) 02 SEPTEMBER 1986, see column 9, lines 7-9 in particular.	1-4,6
Y	US, A, 3,962,439 (YOKOYAMA et al) 08 June 1976, see entire disclosure.	19-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 01 JULY 1996	Date of mailing of the international search report 22 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DR. HERBERT J. LILLING Telephone No. (703)308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03866

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/6, 231.2, 231.5, 231.8, 315, 316 , 451, 461, 747, 755, 759, 788, 832; 530/385